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(54) Title: A MALARIA VACCINE

(57) Abstract

The present invention relates to a substantially pure soluble antigen derived from the malaria parasite *Plasmodium falciparum* in its schizont stage capable of stimulating the production of cytokines, and analogues thereof. The antigen has endotoxin-like properties and stimulates the production of monokines and/or lymphokines, moreover, the antigen is capable of eliciting antibodies wich neutralize the production of cytokines. Furthermore, the present invention relates to antibodies which are reactive with the antigen or an analogue thereof and a method of determining the presence of a *Plasmodium* species molecule in a sample, the sample being incubated with e.g. an antibody which is reactive with the antigen or an analogue thereof, coupled to a solid support. The present invention further relates to the use of said antigen or an analogue thereof for the manufacture of a medicament for the prophylaxis or treatment of diseases caused by *Plasmodium* species, and to a vaccine for immunizing an animal, including a human being, against diseases caused by a plasmodial parasite, the vaccine comprising an immunologically effective and physiologically acceptable amount of said antigen or an analogue thereof. The antigen is an amphiphilic glycoprotein which in a crossed immunoelectrophoresis (CIE) has a position in the γ-globulin fraction and which has fractions of a molecular mass of 77, 61 and 59 kD when tested by SDS-PAGE analysis.

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A MALARIA VACCINE

FIELD OF INVENTION

The present invention relates to an antigen, antigen 7, of *Plasmodium falciparum* or an analogue thereof, an antigen which is recognized by an antibody raised against or reactive with antigen 7, methods of producing and isolating antigen 7 or an analogue thereof, a vaccine comprising said antigen, antibodies directed against antigen 7, T-cell clones reactive with antigen 7, a diagnostic agent comprising said antibodies or said antigen, and the use of such antibodies, T-cell clones or antigen 7 or an analogue thereof for diagnostic and prophylactic and therapeutic purposes, especially in the treatment of the clinical manifestations of malaria.

GENERAL BACKGROUND

Malaria remains one of the most serious diseases in the third world despite the efforts to control the disease and reduce its prevalence and continued geographic spread by vector irradiation and drug treatment. Each year, several hundreds of millions of human beings are affected by malaria. Failure of classic control programmes have stimulated the search for a vaccine approach for the control of malaria.

Human malaria is caused by four species of the protozoean genus, *Plasmodium*. The species *P. falciparum* is the most dangerous, causing acute severe infections that are often fatal, especially in young children and immigrants entering endemic areas. The life cycle of *P.falciparum* includes different stages; in the first stage, the sporozoite stage, the parasite is brought into the blood stream to the liver where they invade the hepatocytes and develop into merozoites in the course of 5-7 days. Merozoites released from infected cells start a new cycle by invading the erythrocytes. It is the erythrocytic stages of the parasite which gives rise to the clinical disease. In the erythrocyte, the parasite shows an asexual multiplication which involve a maturation of the parasite through different parasite stages, the ring, the trophozoite and the schizont

stage (the stage that undergoes nuclear division). When the schizont infected erythrocyte bursts, new merozoites are released. Some merozoites, however, differentiate into micro or macro gametocytes, the sexual forms of the parasite. Contrary to the asexual infected erythrocytes, these sexual parasite stages are able to continue the life cycle, when the infected erythrocytes are ingested by mosquitoes during a blood meal. By fertilization in the mosquito gut, the gametocytes develop into a mobile ookinete stage. The ookinete pass through the epithel and matures into a oocyst. In the oocyst, the new sporozoites develop. These sporozoites are released and move to the salivary gland, and are then ready to be injected into a new host. The parasites are haploid in most of the life cyclus as they perform a meiotic cell division shortly after fertilization. The *Anopheles* mosquito is the primary vector of malaria, but the disease can be seen after blood transfusion, i.v. injection of medicaments and after transfer from an infected mother to the newborn child through the placenta.

The disease malaria is caused by the destruction of erythrocytes, by the adherence of infected erythrocytes to endothel cells in venules, and by a spectrum of disease manifestations most probably caused by the liberation of endotoxins from the parasite (Clark I A, 1987). A natural immunity against malaria is obtained only after several years with repeated infections with the malaria parasite. First, an immunity against the mortality of a malaria infection is established. Subsequently, an immunity is established, which protects against the the clinical manifestations of malaria, and finally, a sterile immunity is established, which protects against infection with the malaria parasites. However, the latter form of immunity is seldom completely effective. Moreover, the immunity is unstable and is lost if the infected person is not regularly subjected to new parasite exposures.

Traditionally, all efforts to develop a vaccine against malaria has been directed to the latter form of immunity, i.e. the immunity which protects against infection with the malaria parasite. It has, however, proven difficult to develop a vaccine providing immunity against invasion by the parasite because the protective antigens have a poor

immunogenicity, *inter alia* because of the existence of multiple antigens, each of which is specific for a given parasite species and a given parasite stage. Furthermore, the parasite has developed evasion mechanisms in order to survive the reactants of the immune system. Thus, it has proven difficult to develop a generally applicable vaccine which provides an immunity against any malaria parasite. Furthermore, the immunity caused by the vaccine should be better than the naturally obtained immunity.

The search for suitable constituents of a malaria vaccine has involved analysis of and search for various *P. falciparum* antigens, and a number of soluble antigens of *P.falciparum* have been found in sera from malaria infected individuals (Jepsen S and Axelsen N H, 1980). Mixtures of soluble antigens have also been isolated from *in vitro* cultures of *P.falciparum* as described in Jepsen S and Andersen B J (1981). The antigens have been defined by the crossed immunoelectrophoresis technique, and each antigen have been designated a number. These antigens have, however, not been described in an isolated or purified form, but only as constituents of a mixture of antigens. Thus, it has not been possible to establish their individual properties and function, if any, during a malaria infection. By the present inventors, a soluble antigen of *P.falciparum* has now isolated and purified, and biological and immunological properties thereof has been identified. It has been found that the isolated antigen and fractions thereof possess surprising properties with respect to antigenicity and immunogenicity which properties may be useful for profylactic and diagnostic purposes in regard of a malaria infection.

BRIEF DISCLOSURE OF THE INVENTION

More specifically, the antigen derived from the malaria parasit *P. falciparum* which has now been isolated and analyzed has been shown to be capable of stimulating the production of cytokines and may be of importance for both pathological events and for the stimulation of the immune system during a malaria infection. Accordingly, the pre-

sent invention relates to a substantially pure soluble antigen derived from the malaria parasite *P. falciparum* in its schizont stage capable of stimulating the production of cytokines, which antigen is an amphiphilic glycoprotein which in a crossed immunoelectrophoresis (CIE) has a position in the γ-globulin fraction and which has fractions of a molecular mass of 77, 61, and 59 kDa when tested by SDS-PAGE analysis under conditions substantially as described in Example 1 or an analogue thereof.

The antigen having the properties described above has been designated "antigen 7" by the present inventors. In the following, the term "antigen 7" may be used about an antigen of the invention having the properties stated above.

In accordance with conventionally used terminology, the cytokines produced by stimulation with the antigen of the invention are to be understood as factors which are produced and secreted by cells exposed to the antigen. Examples of such cells are lymphocytes which produce and secrete lymphokines, and monocytes which produce and secrete monokines.

In the present context, the term "analogue" as used in connection with an antigen of the invention is intended to indicate a substance which is capable of stimulating the production of cytokines *in vitro* and *in vivo*, and which may have other characteristics than the above-cited characteristics of antigen 7 as long as the varying characteristics do not have an adverse effect on the cytokine-stimulating properties of the analogue. The analogue of the antigen may show sereological cross reactivity with antigen 7 defined above, i.e. react with antibodies raised against antigen 7. The analogous antigen may e.g. be derived from another *Plasmodium* species or from a microorganism of another species than *P. falciparum* or may be partially or completely of synthetic origin. The term is further intended to mean a subsequence of the antigen capable of stimulating the production of cytokines and a functional equivalent or derivative of the antigen.

The term "subsequence" is intended to indicate a part of an antigen of the invention which is capable of stimulating the production of

cytokines. The subsequence may, for instance, be any fraction of the antigen e.g. any of the fractions of various molecular masses obtained in a SDS-PAGE analysis as mentioned above, or may be a protein or polypeptide part of the antigen being substantially free from other non-proteinaceous or non-polypeptide parts. Also, glycolipid subsequences of an antigen of the invention may be of interest. A particularly interesting subsequence is an antigenic determinant of the antigen i.e. an epitope, which is a part of the antigen capable of stimulating or interacting with immuno-competent cells in order to stimulate the production of cytokines. Also, an epitope reactive with an antibody raised against an antigen of the invention *in vivo* or *in vitro* may be of interest.

The term "functional equivalent" is intended to include all active substances capable of stimulating the production of cytokines in a manner similar to the stimulation obtained by an antigen of the invention. The functional equivalent may be an anti-idiotypic antibody, i.e. a idiotype structure found on immunoglobulins and T-cells, which structure mimicks an antigen of the invention or a subsequence or derivative thereof.

The functional equivalent may be derived from a microorganism of another species than *P.falciparum* or may partially or completely be of synthetic origin. It should be understood that the similarities between antigen 7 and the functional equivalent are qualitative rather than quantitative.

In the present context, the term "substantially pure" is understood to mean that the antigen in question is substantially free from other components, e.g. other antigens which would have an adverse function with respect to immunological properties. Also, the antigen of the invention is substantially free from substances which result from the production and/or recovery of the antigen or otherwise be found together with the antigen. The high purity of the antigen of the invention is advantageous when the antigen is to be used for immunization purposes, e.g. as a vaccine constituent, as unwanted and adverse immune reactions resulting from the presence of other immunogenic components is avoided. The purity of the antigen of the in-

vention can be determined by Western Blot analysis and by Coomassie Brilliant Blue or silver staining of SDS-PAGE gels.

In another aspect, the present invention relates to a vaccine for immunizing an animal, including a human being, against diseases caused by the malaria parasite, which vaccine comprises an immunological effective and physiologically acceptable antigen as defined above or an analogue thereof together with a physiological compatible carrier and adjuvant. The vaccine should be made so as to allow an optimal stimulation of the relevant parts of the immune system. The vaccine is believed to provide protection against the clinical manifestations of malaria without necessarily providing immunity against the malaria parasite itself and thus, the vaccine takes another approach in the malaria profylaxis than the malaria vaccines comtemplated up to now.

In the present context, the term "clinical manifestation" is intented to mean the various symptoms following after infection with a malaria parasite. Symptoms such as fever, nausea, vomiting, abortion, cerebral malaria, head aches, anaemia, thrombocytopenia etc. are found in connection with a malaria infection.

The present invention also relates to an antibody reactive with the antigen of the invention or an analogue thereof. The antibody may be useful for passive protection against the disease malaria and is intended to block endotoxins of the parasites without necessarily stimulating the immune system which will be further described below.

The antibody may also be used for the identification and/or quantification of at least part of the antigen of the invention present in a sample thus making it possible to diagnose infection with *Plasmodium* species and the presence of parasite endotoxins. The sample may be from any part of a living organism. The sample may e.g. be a body fluid or tissue part containing the antigen, e.g. the sample may be a tissue sample such as a biopsy, e.g. a bone marrow tissue sample, a blood sample, urine, a sample of cerebrospinal fluid, serum, plasma, or any product prepared from blood or lymph, secretions or any sample obtained from a human or animal cavity containing an antigen of the

invention or an analogue thereof. Also the sample may be a vaccine or diagnostic or therapeutic agent in which it is desireable to determine the presence and/or quantity of an antigen of the invention or an analogue thereof.

DETAILED DISCLOSURE OF THE INVENTION

As stated above, antigen 7 is an amphiphilic glycoprotein which is synthetized during the schizont stage of the parasite cyclus and processed during the maturation of the parasite. Processed products of the antigen of the invention have a molecular mass of 77, 61, and 59 kDa (established by an SDS-PAGE analysis under conditions substantially as described in Example 1) and are released to the culture supernatant. The precursor of the antigen has been shown, by SDS-PAGE analysis, to be a molecule having a molecular mass of 130 kDa, when tested under conditions substantially as described in Example 1. Besides the fractions of a molecular mass of 77, 61, and 59 kDa, fractions having a molecular mass of 35, 33 and 16 kDa is normally found when antigen 7 is subjected to SDS-PAGE analysis under conditions substantially as described in Example 1. These latter fractions are believed to be processed products of the former fractions. Antigen 7 contains protein, carbohydrate, and liquid components, and the composition of the fractions cited above having various molecular masses is believed to be complex although it has not yet been fully elucidated.

The antigen of the invention is easily recognized in a crossed immunoelectrophoresis which has been found to be the most reproducible manner in which the antigen of the invention can be described. By crossed immunoelectrophoresis (CIE) the entire antigen in its native form is visualized. The crossed immunoelectrophoresis technique may be carried out substantially as described by Jepsen and Axelsen, 1980, and is based on the principle of precipitating the antigen of the invention with an antibody reactive with such antigen. Other ways of identifying an antigen of the invention are described below and comprises, *inter alia*, visualization of an antigen-antibody reac-

tivity based on an enzymatic reaction producing a coloured substrate, i.e. in accordance with the ELISA-technique.

The antigen of the invention has preferably endotoxin-like properties. By the term "endotoxin-like properties" is meant that the antigen of the invention is capable of exerting the same or similar effects as an endotoxin, e.g. a lipopolysaccharide, i.e. a substance which is usually found in gram-negative bacteria, primarily on the outer surface of the bacterial membrane. The endotoxin is usually a pyrogen, i.e. induces fever.

The endotoxin-like properties of the antigen of the invention may be determined in a so-called *limulus amoebocyte lysate* (LAL) assay, e.g. as described in Example 1. The LAL-assay has been found to provide a very sensitive determination of even small amounts of endotoxin. Also, when the antigen of the invention is tested in a LAL-assay, e.g. as outlined in Example 1 below, the antigen is shown to behave like an endotoxin.

The endotoxin-like properties of the antigen of the invention are preferably manifested in their capability of stimulating the *in vitro* or *in vivo* production of monokines such as tumor necrosis factor (TNF) or interleukin-1, or the *in vitro* or *in vivo* production of lymphokines such as gamma-interferon, interleukin-2, interleukin-4 and interleukin-6. The production of monokines and lymphokines may be determined by conventional methods, for instance, interleukin-1 may be determined substantially as described in Example 2 below.

As mentioned above, the antigen of the invention is preferably pyrogenic, i.e. capable of inducing fever in an individual. However, by stimulating the production of tumor necrosis factor (TNF) and interleukin-1, the antigen of the invention is also expected to be involved in the development of other diseases associated with or caused by malaria, e.g. in the development of cerebral malaria and in inhibition of bone marrow development, and optionally in provoking abortion; see Grau G E et al. (1987), Grau G E et al. (1988), Clark I A et al. (1988) and Miller K L et al. (1989).

The present invention also relates to a substantially pure soluble antigen derived from a malaria parasite capable of eliciting antibodies which neutralize the production of cytokines *in vitro* or *in vivo* or an analogue of said antigen. It is expected that the antibodies raised against a substantially pure soluble antigen according to the present invention derived from a malaria parasite is capable of preventing or reducing the clinical manifestations associated with a malaria infection. The antibodies are believed to act either by reacting with malaria antigens circulating in the body of malaria-infected individuals, e.g. in the blood, thereby neutralizing the antigens and preventing the antigens stimulation of cytokine production causing the clinical manifestations of a malaria infection.

Preferably, the antigen capable of raising antibodies neutralizing the production of cytokines is derived from a *Plasmodium* species, and more preferably from *P. falciparum*. Antigen 7 as described above has been found to raise antibodies which neutralize the production of cytokines.

As indicated above, at least two mechanisms exist which are responsible for that the antigen of the invention or an analogue thereof may exert its antigenic or immunogenic effect. Thus, one mechanism is based on the capability of the antigen or an analogue thereof of stimulating the production of cytokines from various cell types, the other mechanism is based on the capability of the antigen to elicit antibodies neutralizing said production of cytokines. The two mechanisms are connected with the B-cell and T-cell system, respectively, which will be further discussed below.

In the T-cell system, the antigen of the invention or an analogue thereof induces a proliferative response in a T-lymphocyte. The term "proliferative response" is understood to mean that the T-lymphocyte responds to an exposure of an antigen of the invention or an analogue thereof, e.g. a subsequence thereof such as an antigenic determinant, i.e. an epitope, presented in the antigen, by producing interferon and interleukin, which are substances being capable of eliciting an antibody production in B-lymphocytes of the immune system. The proliferative response may be determined by detecting and optionally

quantifying the interferon and interleukin produced upon the exposure, or by allowing the interferon or interleukin produced to elicit the antibody production from B-lymphocytes and determining the presence and/or amount of the resulting antibodies. As it will be discussed below, an antigen determinant eliciting a proliferative response in T-lymphocytes, i.e. a T-cell epitope, may advantageously be used in combination with antigen determinants being recognized by antibodies produced by the B-lymphocytes (B-cell epitope) for immunization purposes. The B-cell and T-cell epitopes are further explained below.

In the B-cell system, "B-cell epitopes" of the antigen or an analogue thereof elicit the production of antibodies reactive with the antigen. By the term "B-cell epitopes" is meant the structures in an antigen which interact specifically with the variable part of an immunoglobulin and thus, the B-cell epitopes are recognized by antibodies produced by the B-cells. The B-cell epitope may be a glycopeptide comprising a lipid and/or a carbohydrate part or any of these, or a polypeptide, the structure of which may be a stretch of amino acids in the primary amino acid sequence of the polypeptide part of the antigen, or a group of amino acids which are brought spatially together from parts of the amino acid sequence which are not contiguous in the primary sequence, e.g. by means of the secondary or tertiary structure of the polypeptide part of the antigen. Usually, B-cell epitopes having a polypeptide structure contain a rather small number of amino acids, e.g. comprising from about 3 to about 20 amino acids, more usually from about 4 to about 12 amino acids.

The term "T-cell epitopes" is to be understood as the structures in the antigen which are presented by antigen-presenting cells and which interact with the T-cell receptor. The interaction between the antigen-presenting cells (i.e. macrophages, B-cells, dendritic cells, interdigitating cells and Langerhans cells) and the T-cell receptor is supposed to be mediated in the following manner: The antigenpresenting cells internalize the antigens by endocytosis or pinocytosis and subsequent processing by proteolytic cleavage of the antigens to smaller fragments, whereby the interaction between the antigen pre-

senting cells and the T-cell receptor is established. The T-cell spitope may be any part of the antigen, e.g. a glycopeptide as descussed above, or a non-glyco-sylated polypeptide. When the T-cell epitope comprises a polypeptide, the processing has been shown to include proteolytic cleavage of the primary structure which produces fragments of the 8-20 amino acids which very often have an amphifilic α-helical structure. Other alternative ways of processing are evident as it has been shown that a T-cell epitope might be composed of non-contiguous amino acids of the primary structure of the polypeptide part of the antigen. The amphifilic α-helices are then presented on the external cell surface in relation to the molecules of the major histocompatibility complex (class II). The complex of the amphifilic α-helical peptide from the antigen and the histocompatibility molecule is then recognized by T-cells specific for this antigen and triggers the production of lymphokines, growth factors, differentiation factors and some of the corresponding receptors. These substances stimulate the B-cells to produce antibodies against B-cell epitopes related to the T-cell epitope and stimulates natural killer cells (NK cells), killer cells, macrophages and cytotoxic T-cell to engage targets presenting the antigen. Thus, the T-cell epitopes do not in themselves produce antibodies but elicit the antibody production from B-cells and are not necessarily recognized by the antibodies raised against antigen 7.

The analogue of the antigen of the invention may solely comprise T-cell epitopes or solely B-cell epitopes or a combination of these. Thus, the composition or an analogue of the antigen of the invention thereof may be tailored to its intended use, e.g. its use as a vaccine component. As discussed above, T-cell epitopes are extremely advantageous as they enhance and accelerate the immune response and the production of antibodies. Furthermore, the memory function of the immune system resides in the T-cells. B-cell epitopes are advantageous for most applications as they are required for eliciting an antibody production. Antibodies may bind to endotoxin-like antigens of the parasites and neutralize the toxin activity which, through the stimulation of cytokines, e.g. TNF and interleukin-1, may be wholly or partially responsible for fever, cerebral manifestations, anaemia, abortion, etc. during a malaria infection. However, the antibodies

may also bind to the malaria parasite and neutralize it directly or together with complement, phagocytotic and cytotoxic cells.

The antigen defined above is processed into fractions of the various molecular masses outlined above as determined under the conditions outlined in Example 1 below. As it will be explained in further details below, preliminary experiments indicate that the fraction having a molecular mass of 16 kDa is particularly immunogenic.

The antigen described above is a amphiphilic glycoprotein being constituted of protein, lipid and carbohydrate components. Antigenic determinants of antigen 7 may be situated in various parts of the glycoprotein. Thus, the antigenic determinants may be found in fractions of the protein having a complex structure (i.e. as is the case with the 16 kDa fragment mentioned above which is a glycolipid). However, the antigenic determinants may also reside in small fragments of the glycoprotein being constituted of only one type of component. In accordance herewith, the present invention also relates to a substantially pure polypeptide derived from an antigen of the invention. The polypeptide should be capable of stimulating the production of cytokines and/or elicit antibodies which neutralize the production of cytokines *in vivo* or *in vitro* in order to effect the desired antigenic and immunogenic properties. A polypeptide part of an antigen of the invention is advantageously produced as a recombinant or synthetic polypeptide. When the polypeptide does not comprise lipid or carbohydrate parts, it may rather easily be produces by microorganisms in accordance with methods conventionally used within recombinant DNA-technology. Also, when the polypeptide is not too long, it may also easily be produced by peptide synthesis. This will be dealt with in further details below.

The antigen of the invention or an analogue thereof may be in the form of a fusion protein in which one or more polypeptide parts of an antigen as defined above having B-cell and/or T-cell stimulating properties is/are fused to another polypeptide sequence. The fusion protein may be in glycosylated from. Typically, a fusion protein will be prepared by recombinant DNA-techniques or peptide synthesis. Methods for the preparation of a fusion protein will be futher desc-

ribed below. The polypeptide to which the polypeptide part of antigen 7 is/are fused may be one which results in an increased expression of the fusion protein by the organism when expressed in an organism, or facilitates or improves the purification and recovery of the fusion protein from said organism in terms of a more easy and economical recovery. Further, a peptide sequence which increases the immunogenicity may advantageously be coupled to a polypeptide part of an antigen of the invention so as to adapt the resulting fusion protein for vaccine components. An example of such peptide sequence is, for instance, another parasite antigen, e.g. an antigen derived from a *Plasmodium* species, e.g. *P. falciparum*. For instance, a *P. falciparum* antigen as described in US patent application, Serial Number 318,885, filed on 3 March, 1989, which patent application is incorporated herein by reference, may be used.

In some cases, after the production of a fusion protein it may be advantageous to cleave the fusion protein so as to obtain a polypeptide which substantially solely comprises one or more polypeptide part(s) of antigen 7. In these cases, polypeptide part(s) of antigen 7 is/are preferably fused to a polypeptide sequence which may be specifically recognized by a cleaving agent, e.g. a chemical such as cyanogen bromide, hydroxylamine and 2-nitro-5-thiocyanobenzoate, or an enzyme, e.g. a peptidase, proteinase or protease, e.g. trypsin, chlostripain, and staphyllococal protease.

The polypeptide to which one or more polypeptide part(s) of antigen 7 is/are fused may be one which modifies the immunogenic properties of the fusion protein and may comprise a polypeptide sequence which is easily detectable, e.g. by means of antibodies directed against said sequence or by means of a specific chemical or enzymatic reaction.

Further, the antigen of the invention or an analogue thereof may be coupled to a carbohydrate or a lipid moiety, e.g. a carrier, or modified in other ways, e.g. being acetylated.

The variant domain of an antibody is composed of variable and constant sequences. The variant part of the domain is called the idioty-

pe of the antibody. This part of the antibody is responsible for the interaction with the antigen, the antigen binding.

The idiotypic structure is antigenic and can thus give rise to specific antibodies directed against the idiotypic structure. This has been done in mice. The antibodies raised against the idiotype, the anti-idiotypic antibodies, may mimic the structure of the original antigen and therefore may function as the original antigen to raise antibodies reactive with the original antigen. This approach may be advantageous as it circumvents the problem associated with the characterization and synthesis of the important immunogenic parts of the antigen in question. This is most important in the case of conformational epitopes, which might otherwise be difficult to identify. It has been shown for a number of organisms that protective immunity can be induced in this way (e.g. *Trypanosoma cruzi*, *Trypanosoma brucei*, Hepatitis B virus, and *Plasmodium knowlesii*). In accordance herewith the present invention also relates to an anti-idiotypic antibody mimicking the structure of an antigen of the invention or an analogue thereof and capable of stimulating T-cells and antibodies reactive with the antigen or the analogue.

The antigen of the invention or an analogue thereof, e.g. a polypeptide part thereof or an anti-idiotypic antibody mimicking the structure of the antigen or analogue, is contemplated to be useful as a prophylactic or therapeutic agent, e.g. a vaccine, and may be used in the manufacture thereof. This will be explained below.

The present invention also relates to a vaccine for immunizing an animal, including a human being, against diseases caused by a plasmodial parasite, which vaccine comprises an immunologically effective and physiologically acceptable amount of an antigen of the invention or an analogue thereof, together with a physiologically compatible carrier or vehicle.

The term "vaccine" is to be understood to comprise any preparation containing an antigen of the invention or an analogue thereof suited for administration to living organisms for the prevention, amelioration or treatment of the clinical manifistations of a *Plasmodium*

species infection, and optionally of the infection as well. Preferably, the plasmodial parasite is a *P. falciparum*. The term "immunization" is understood to comprise the process of evoking a specific immunologic response with the expectation that this will result in humoral, and/or secretory, and/or cell-mediated immunity to infection with *Plasmodium* species, i.e. immunity is to be understood to comprise the ability of the individual to resist or overcome clinical manifestations of a malaria parasite infection, i.e. to tolerate the infection without being clinically affected, or to overcome clinical manifistations "more easy" compared to individuals who have not been immunized. Thus, the immunization according to the present invention is a process of increasing resistance to clinical manifistations of an infection with *Plasmodium* species. An overall aspect in the preparation of the vaccines of the invention is the physiological acceptability of the components and of the total composition of the vaccine. The final formulation of the vaccine should be a mixture of substances supporting and enhancing the immune response induced by the specific immunogenic component.

In a preferred embodiment of the invention a vaccine is developed which is strain-non-specific, i.e. it comprises an epitope or epitopes which are protective common to substantially all strains of the *Plasmodium* falciparum causing infections of considerable clinical importance. In this case, an epitope according to the present invention being conserved in different *Plasmodium* species is advantageous.

In another preferred embodiment of the invention, a multivalent vaccine is formulated, i.e. several immunologically effective components are incorporated into a single vaccine being effective in reducing clinical manifistations associated with a *Plasmodium* species infection, reducing infection and/or transmission - all in all inducing an effective protective immunity. The vaccine may comprise one or more additional molecules which are not related to an antigen of the invention or an analogue thereof in order to providing the multivalent nature of the vaccine. Especially interesting additional molecules are immunologically active molecules obtained from pathogenic organisms other than *Plasmodium* species organisms which give rise

to a vaccine being effective in reducing infection or disease manifestations caused thereby or providing immunity for one or more pathogenic organisms in addition to the malaria parasite.

In the production of the various vaccine types the use of antigens or analogues thereof produced by recombinant DNA techniques or synthetically, e.g. as will be described below, is preferred. A major advantage of this strategy is the ability to produce an unlimited amount of a purified product and the avoidance of contamination by pathogens. Routine methods for vaccine production involve risks of obtaining unwanted side effects, e.g. due to the vaccine containing unwanted (or even unidentified) contaminants. The methods of preparation of vaccines according to the present invention should be designed to ensure that the identity and immunological effectiveness of the specific molecules are maintained and that no unwanted microbial contaminants are introduced. The final products are distributed under aseptic conditions into preferably sterile containers which are then sealed to exclude extraneous microorganisms.

The vaccine may further comprise an adjuvant in order to increase the immunogenicity of the vaccine preparation. The adjuvant may serve the purpose of enhancing the stimulatory properties of the antigen of the invention or an analogue thereof by stimulating the production of cytokines or lymphokines from the cells of the immune system in a non-specific way and the adjuvant may trap the antigen or analogue to avoid degradation. The adjuvant may be selected from the group consisting of Freund's incomplete or complete adjuvant, aluminium hydroxide, a saponin, a muramyl dipeptide, a lipopolysaccharide, an immunogenic part of other microorganisms, a T-cell immunogen, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6 interferon-gamma, an oil, such as a vegetable oil, e.g. peanut oil, or a mineral oil, e.g. silicone oil, purified protein derivatives (PPD), and Bacille Calmet Guerin (B.C.G.).

Another vaccine form is contemplated to be useful as it improves the transportation of the vaccine and the physical-chemical presentation, and prolongs the time of presentation for the relevant parts of the immune system. Such vaccine comprises a vehicle which may be in

various forms. The vaccine may comprise an antigen of the invention or an analogue thereof incorporated into micelles, (using micelle-forming agents such as detergents, preferably non-ionic detergents or other non-denaturating micelle-forming agents such as amphiphilic peptides, glycosides), open spherical structures, consisting of circular subunits or parts of spherical structures, the formation of which utilizes the hydrophobic/hydrophilic properties of the antigen of the invention or an analogue thereof. Also vaccines are contemplated in which the antigen of the invention or an analogue thereof is incorporated into so-called iscoms (immune stimulating complexes, as disclosed, e.g., in EP 0 109 942).

The antigen of the invention or an analogue thereof may advantageously be coupled to a carrier, which may be any carrier usually employed in the preparation of vaccines. The carrier may be a macromolecular carrier, e.g. comprising polysaccharides or polypeptides to which the antigen of the invention or an analogue thereof is covalently or non-covalently bound. The carrier should preferably be non-toxic and non-allergenic. The antigen of the invention or an analogue thereof may be multivalently coupled to the macromolecular carrier as this provides an increased immunogenicity of the vaccine preparation. In this regard, it may prove advantageous to couple the antigen of the invention or an analogue thereof to the carrier together with one or more immunologically active molecules obtained from organisms other than plasmodium species so as to obtain a vaccine comprising a variety of different immunogenic determinants, i.e. a cocktail vaccine, which may be employed for the immunization of diseases caused by a variety of different organisms. A vaccine, wherein the antigen of the invention or an analogue thereof is polymerized, i.e. so as to present the antigen of the invention or an analogue thereof in a multivalent form, may also prove advantageous.

Various immunization schedules may be employed when using the vaccine of the invention: In some instances it may be appropriate to provide active immunization early in life. Furthermore, it may be desirable to employ repeated administrations, e.g. at regular or prolonged intervals. Any immunization schedule which may be contemplated or

shown to produce an appropriate immune response can be employed in accordance with the principles of the present invention.

The vaccine should be administered in a way which ensures an efficient stimulation of the immune system. This means that the vaccine should be brought into contact with the cells of the immune system for a sufficient period of time and in a form capable of functioning as an immunogen. Several ways are possible. Of these the most conventional are the parenteral ways, i.e., the subcutaneous, intradermal, intramuscular or the intravenous route.

Other more unusual ways of administering the vaccine are the nasal, the oral or the rectal routes. A combination of the two, first mentioned routes could be achieved by using an aerosol formulation of the vaccine to be administered via the respiratory tract. This formulation of a vaccine has been proposed for special purposes where a more conventional formulation would be insufficient due to: the need for vaccination in very remote areas, the logistic problems associated with transportation and storage of the vaccine, problems associated with infections being spread by multiple use of syringes and the need to vaccinate large populations.

The aerosol vaccine is in most cases administered via the nasal route. It is known that peptides can be transported intactly through the nasal mucosa to reach the blood. When transported further down the respiratory tract, the antigen is taken up by the macrophages functioning as scavengers and is in this way potentially presented to the immune system. Some of the material administered as an aerosol may possibly reach the intestines and stimulate the immune system present in the intestines and this way stimulate the immune system of the body, or may be taken up by the intestinal mucosa in intact form and liberated to the blood stream where it will be presented for the immune system.

The vaccine may also be administered strictly via the nasal route. This way simplifies the administration and circumvents the problems associated with spreading of infectious diseases through multiple use of syringes.

The oral route of administrating the vaccine utilizes the finding, that certain proteins are taken up by the intestinal mucosa and are found in intact form in the bloodstream. This special way of administering the vaccine will take advantage of pharmaceutical formulations protecting the immunogenic components from degradation in the stomach or in the intestines. An effect of administrating the vaccine via the oral route may also come from the antigen of the invention or an analogue thereof stimulating that part of the immune system which is residing in the intestines and in the liver - and this way leading to a general immune stimulation.

The rectal route of administering the vaccine has the same advantages as the above mentioned methods but might be more reliable and thus lead to greater patient compliance in special groups, i.e. children.

When the DNA sequence encoding an antigen of the invention or an analogue thereof has been elucidated it may be possible to use the DNA sequence for the preparation of certain types of vaccines. One interesting vaccine is a live vaccine, i.e. a non-pathogenic microorganism which carries and is capable of expressing an inserted nucleotide sequence coding for an antigen of the invention or an analogue thereof, which microorganism is of a type which is suitable as a live vaccine for the immunization of an animal against diseases caused by malaria parasites. The use of a live vaccine might be advantageous since there is some indication that vaccines based on living organisms show an excellent immunogenicity, often conferring a lifelong immunity against the disease in question. Live vaccines also tend to be less expensive to produce than those based on a purified antigen, no purification step being required. The antigen of the invention or an analogue thereof may advantageously be expressed on the outer surface of the non-pathogenic organism. This provides a favorable presentation of the antigen of the invention or an analogue thereof which will be recognized by the immune defense mechanisms of the animal to which the live vaccine is administered.

Alternatively, one or more DNA sequences encoding antigens could be inserted into a virus genome, e.g. into a retrovirus, vaccinia,

Epstein-Barr virus genome, to produce a polyvalent vaccine. A DNA sequence encoding an antigen of the invention or an analogue thereof, e.g. an immunologically equivalent or derivative thereof, could be recombined with vaccinia to yield a vaccine to protect against clinical manifistations of, or infection with *Plasmodium* species.

Furthermore, passive immunization may be employed, i.e. an administration of a preparation containing antibodies, e.g. of the type described below, especially a preparation with a high content of purified antibodies, to the patient to be protected against malaria.

As stated above, the DNA sequence or gene encoding an antigen of the invention as defined above or an analogue thereof has not yet been elucidated. However, as it is described in Example 4, 5 positive clones have been obtained from a *P. falciparum* gene library, which clones react with antigen 7 monospecific sera from malaria immune adults, indicating that the clones express antigen 7. The isolation of the gene or DNA sequence encoding antigen 7 has not yet been accomplished but it is believed that the isolation of the DNA sequence and subsequent sequencing may be carried out, in accordance with well-known methods, e.g. as described by Maniatis et al (1982), substantially as described in Example 4 below.

More specifically, the DNA encoding an antigen of the invention or an analogue thereof may be of complementary DNA (cDNA) origin, that is, obtained by preparing a cDNA library on the basis of mRNA from cells producing the antigen or an analogue thereof, i.e. from cell of a malaria parasite preferably of the genus *Plasmodium*, and more preferably of the species *P. falciparum*, by means of established standard methods and vectors. Antibody reactivity using antibodies, preferably monospecific or monoclonal antibodies recognizing clones expressing the antigen or an analogue thereof, may then be used to identify the clone containing the cDNA sequence encoding the antigen or an analogue thereof. Alternatively, exposure of cells capable of producing cytokines to a clone expected to contain the DNA sequence encoding the antigen or an analogue thereof and determining any cytokine production. When the DNA sequence encoding an antigen of the invention has been elucidated, it will be possible to identify

clones harbouring a cDNA sequence encoding an antigen of the inevention or an analogue thereof by hybridization experiments using DNA probes, e.g. synthetic oligonucleotides. Alternatively, the DNA sequence may be of genomic origin, that is, derived directly from a cellular genome, for instance by screening for genomic sequences hybridizing to a DNA probe or by antibody reactivity. This is illustrated in Example 4. Genomic DNA differs from cDNA, for instance by containing transcriptional control elements and non-coding regions, e.g. introns, found within the coding DNA sequence.

The DNA sequence may also be of synthetic origin, i.e. prepared synthetically by established standard methods, or of mixed synthetic and genomic origin, mixed genomic and cDNA origin or mixed cDNA and synthetic origin prepared by ligating together DNA fragments of cDNA, genomic or synthetic origin (as appropriate), which DNA fragments comprise part of the gene encoding the antigen or an analogue thereof, in accordance with standard techniques.

The DNA sequence encoding antigen 7 or an analogue thereof has a number of applications. Thus, the DNA sequence may be used in the recombinant production of antigen 7 or an analogue thereof allowing for the production of large amounts of antigen 7 or an analogue thereof, in particular in a substantially pure form, free from contaminating substances related to *P. falciparum* parasites or serum from infected individuals, from which antigen 7 hitherto has been recovered. The recombinant production may be carried out in accordance with standard techniques, i.e. by inserting the DNA sequence in a suitable vector, transforming the vector into a suitable host organism, cultivating the host organism so as to produce antigen 7 or an analogue thereof, and subsequently recovering the antigen from the host organism. The DNA sequence encoding antigen 7 or an analogue thereof may also be used for diagnosis, e.g. for detecting *P. falciparum* DNA in a sample hybridizing to the DNA sequence, e.g. by using conventional hybridization methods such as using a labelled DNA molecule for the detection, or by more specialized methods, e.g. using the principles of polymerase chain reaction. Furthermore, a DNA sequence encoding an antigen of the invention or an analogue thereof may be the active component in a drug for the treatment of malaria

parasite infection, especially infection by a *Plasmodium* species parasite such as *P. falciparum*, and may be formulated for suitable administration forms, e.g. for oral or parenteral administration.

Furthermore, by knowing the nucleotide sequence of DNA encoding an antigen of the invention or an analogue thereof it is possible, by use of computer analysis, to identify advantageous regions of the sequence, e.g. regions encoding epitopes or other highly immunogenic or antigenic regions. After identification of such regions, each of these may be prepared separately, e.g. by use of recombinant DNA technology in accordance with standard methods, and used for the preparation of a vaccine or used for highly specific diagnosis.

To analyze the potential importance of different T-cell epitopes of an antigen of the invention, T-cell clones reactive with the antigen could be produced according to well-known methods, e.g. the method outlined in Example 14 below. The T-cell clones may be used for the screening of recombinant proteins, synthetic peptides, or anti-idiotypic antibodies for the abundance of T-cell epitopes. The T-cell clones can be tested for their production of lymphokines such as γ-interferon, interleukin-2, interleukin-4, interleukin-6, etc. The production of lymphokines indicates the T-cell epitope or epitopes having valuable immuno-stimulating properties. It is anticipated that T-cell clones directed against analogues of antigen 7 in rodent malaria parasites may be tested *in vivo* for their ability to protect rodents against a clinical disease during a rodent malaria infection. Such data would elucidate the parts of antigen 7 useful in a malaria vaccine.

The DNA sequence encoding the antigen of the invention or an analogue thereof may be fused to another DNA sequence encoding another polypeptide with the purpose of construction a DNA sequence encoding fused polypeptide, e.g. as discussed above. The DNA sequences to be fused is preferably in the same reading frame allowing for expressing of the fused sequence. When using recombinant DNA technology, the fused sequence may be inserted into an appropriate vector which is transformed into a suitable host microorganism. Alternatively, the DNA fragment of the invention may be inserted in the vector in frame

with a gene carried by the vector, which gene encodes a suitable polypeptide. The host microorganism is grown under conditions ensuring expression of the fused sequence after which the fused polypeptide may be recovered from the culture by physico-chemical procedures, and the fused polypeptide may be subjected to gel filtration and affinity chromatography using an antibody directed against the antigenic part(s) of the fused polypeptide. After purification, the polypeptide of the invention and the polypeptide to which it is fused may be separated, for instance by suitable proteolytic cleavage, and the polypeptide of the invention may be recovered, e.g. by affinity purification or another suitable method.

The DNA sequence encoding an antigen of the invention or an analogue thereof may also comprise a suitable nucleotide sequence controlling the expression and replication of the DNA fragment. The regulatory nucleotide sequence is conveniently a part of the expression vector used for the production of the polypeptides, when such a vector is employed.

As stated above, the antigen of the invention is a amphiphilic glycoprotein having protein, lipid and carbohydrate components. Therefore, to produce the entire antigen or a part thereof comprising the various components it is necessary to produce it by methods which allow for the production of the rather complex antigen. Of course, the antigen could be obtained from the malaria parasite itself using suitable recovering and purification procedures, e.g. as explained in Example 1 below. However, such procedures do not easily lead to a high amount of the antigen or analogue thereof and would not be suitable for large scale production.

Conveniently, the antigen or an analogue thereof is produced by recombinant DNA techniques. Even though microorganisms such as bacteria may be used for the production, especially of polypeptide analogues of the antigen, it is contemplated that mammal cells may be more advantageous, because in most cases mammal cells will effect glycosylation of the proteins they produce, thereby making the production of the rather complex antigen of the invention possible. A more detailed explanation of the production of an antigen of the

invention by a mammalian cell is given in Example 4 below. Higher organisms such as animals, may be useful for the transgenic production of an antigen of the invention.

The production of the antigen of the invention or an analogue thereof using recombinant DNA techniques may be carried out in accordance with well known methods within the art, e.g. comprising the following steps.

a) isolating a DNA-fragment encoding an antigen as defined above or an analogue therof,

b) inserting the DNA-fragment obtained in a) in an expression vector,

c) transforming af suitable host cell with the vector produced in step b),

d) cultivating the cell produced in step c) under suitable conditions for expressing the antigen or analogue or a post-translationally modified antigen or analogue, and

e) recovering the antigen or analogue or the modified antigen or analogue from the culture.

More specifically the method may be carried out as follows:

Firstly, the DNA sequence encoding the antigen or an analogue thereof to be produced should, of course, be provided either from the malaria parasite itself, e.g. as described above, or from a clone containing the DNA. When the DNA sequence encoding the antigen or an analogue thereof has been elucidated, the DNA sequence may be prepared by DNA synthesis in accordance with conventional procedures.

The DNA sequence is then introduced into the production organism or cell. This may be accomplished by directly inserting the DNA sequence into the genome of the organism or the cell. Alternatively and preferably, the DNA sequence is introduced into the organism or cell by use of a vector carrying the DNA sequence. The vector may by any

vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication; examples of such a vector are a plasmid, phage, cosmid, mini-chromosome and virus. Alternatively, the vector may be one which, when introduced in a host cell, is integrated in the host cell genome and replicated together with the chromosome(s) into which it has been integrated. The construction of a vector carrying the DNA sequence and the introduction of the vector or the DNA sequence into the organism may be accomplished in accordance with well known techniques within the art, e.g. as described by Maniatis et al (1982) and Colbere-Garapin F et al (1981).

The organism or cell harbouring the DNA sequence is then grown under suitable conditions allowing for the expression of the DNA sequence and suited to the organism in question. For instance, in the case of mammal cells a conventional growth medium may be employed and the growth conditions such as temperature, pH, aeration and micro and macro nutrients should of course be adapted to the cell in question as well as to the product to be expressed.

Subsequently, the antigen of the invention or an analogue thereof is recovered from the organism. When required, the organism is ruptured so as to release the antigen or analogue which is then recovered from the ruptured cell or cell culture by conventional methods, e.g. by extraction, centrifugation, affinity or size chromatography, ion exchange chromatography, gel filtration, or other conventionally used a isolation and purification methods.

More specifically, an antigen of the invention or an analogue thereof may be isolated from a biological material containing the antigen or an analogue thereof, e.g. a suspension of cells producing the antigen or an analogue thereof, by use of a method comprising adsorbing the biological material to a matrix comprising an immobilized monoclonal or polyclonal antibody as described herein, eluting the antigen or an analogue thereof from the matrix, and recovering the antigen of the

invention or an analogue thereof from the eluate. Examples of procedures for isolating the antigen or an analogue thereof are:

a) A procedure employing antibodies reactive with *Plasmodium* species molecules which is suited for the obtainment of a *Plasmodium* species containing fraction with high purity, especially a fraction which contains molecules of the species *P. falciparum*, especially in the schizont stage. The procedure may be performed by immobilizing the specific antibodies, preferably monoclonal antibodies, to a matrix, contacting said matrix with the preparation containing the released *Plasmodium* species molecules, washing, and finally treating the antigen-antibody complex fixed to the matrix so as to release the *Plasmodium* species molecules in a purified form. A preferred way is to isolate the *Plasmodium* species molecules by means of column affinity chromatography involving antibodies fixed to the column matrix.

b) Procedures involving various forms of affinity chromatography, gel filtration, ion exchange or high performance liquid chromatography (HPLC).

c) Preparative electrophoresis procedures; for instance the following procedure: A supernatant from a centrifuged enzyme treated cell or cell line preparation is subjected to a gel electrophoresis, such as a sodium dodecyl sulphate-polyacrylamidgel electrophoresis (SDS--PAGE) (cf. Laemmli, 1970), e.g. as described in Example 1? below, or an agarose gel electrophoresis. Subsequently, labelled antibodies, such as monoclonal antibodies, reactive with *Plasmodium* species, are used to identify bands primarily constituted by the isolated *Plasmodium* species molecules. For instance, the antibodies may be used in any conventional immunoblotting technique. The markers may be isotopes or fluorescein labels detectable by means of relevant sensitive films. After identification, the *Plasmodium* species containing bands of the gel may be subjected to a treatment resulting in the release of the *Plasmodium* species molecules from the gels, such as procedures involving slicing up the gel and subsequent elution of *Plasmodium* species molecules. The composition and optionally the amino acid

sequence of the *Plasmodium* species molecules obtained may be determined.

The isolation procedures, discussed above, employing antibodies raised against or being reactive with an antigen of the invention or an analogue thereof may, besides being effective for the isolation procedure, be used to identify parts of the antigen of the invention being of particular interest for immunization and/or diagnostic purposes. Thus, for instance, subjecting the processed products of an antigen of the invention, e.g. obtained from a malaria parasite such as *P. falciparum*, to SDS-PAGE electrophoresis and determining the reactivity of each of the fractions obtained with a suitable antibody, preferably a monospecific or monoclonal antibody, e.g. as described herein, may elucidate the fractions which have the highest reactivity with the antibody and thus may be the best candidates for immunizing against the clinical manifistations of malaria. It should, however, be noted that parts of the antigen of the invention which do not react positively or only weakly with antibodies against the antigen or analogue, may be very efficient stimulators of cytokines and of T-lymphocytes production and *vice versa*, indicating the different function of various parts of the antigen. Thus, the 16 kDa fraction of antigen 7 has been shown to stimulate a high production of TNF without showing any substantial reactivity with antibodies against antigen 7, cf. Example 2.

Prior to cultivation of the microorganism, the DNA fragment encoding an antigen of the invention or an analogue thereof may be subjected to modification, before or after the DNA fragment has been inserted in the vector. The antigen of the invention or an analogue thereof may also be subjected to modification. The modification may comprise substitution, addition, insertion, deletion or rearrangement of one or more nucleotides or amino acids in the DNA fragment or the antigen of the invention or an analogue thereof, respectively, or a combination of these modifications. The term "substitution" is intended to mean the replacement of any one or more amino acids or nucleotides in the full amino acid or nucleotide sequence with one or more others, "addition" is understood to mean the addition of one or more amino acids or nucleotides at either end of the full amino acid or nucleo-

tide sequence, "insertion" is intended to mean the introduction of one or more amino acids or nucleotides within the full amino acid or nucleotide sequence, and "deletion" is intended to indicate that one or more amino acids or nucleotides have been deleted from the full amino acid or nucleotide sequence whether at either end of the sequence or at any suitable point within it. "Rearrangement" is intended to indicate that one or more amino acids or nucleotides of the sequence has been exchanged with each other. The DNA fragment may, however, also be modified by subjecting the organism carrying the DNA fragment to mutagenization, preferably site directed mutagenization so as to mutagenize said fragment. When the organism is a microorganism, the mutagenization may be performed by using conventional mutagenization means such as ultraviolet radiation, ionizing radiation or a chemical mutagen such as mitomycin C, 5-bromouracil, methylmethane sulphonate, nitrogen mustard or a nitrofuran or mutagens known in the art, e.g. mutagens of the type disclosed in Miller JH (1972).

Examples of suitable modifications of the DNA sequence are nucleotide substitutions which do not give rise to another amino acid sequence of the protein, but which, e.g., correspond to the codon usage of the specific organism in which the sequence is inserted; nucleotide substitutions which give rise to a different amino acid sequence and therefore, possibly, a different protein structure without, however, impairing but rather improving the critical properties of the antigen of the invention or an analogue thereof encoded by the DNA sequence; a subsequence of the DNA sequence shown above encoding an antigen of the invention or an analogue thereof which has retained or improved the antigenic immunogenic properties of the native Antigen 7; or a DNA sequence hybridizing to at least part of a DNA malecule prepared on the basis of the DNA sequence shown above, provided that it encodes an antigen of the invention or an analogue thereof which has the biological property of antigen 7.

The antigen of the invention or an analogue thereof produced as described above may be subjected to posttranslational modifications such as for instance thermal treatment, treatment with a chemical

such as formaldehyde, glutar aldehyde or a suitable proteolytic enzyme, e.g. a peptidase or proteinase, such as trypsin.

The posttranslational modification of the antigen of the invention or an analogue thereof may serve the purpose of improving the critical properties of the antigen or analogues, e.g. its antigenic immunogenic properties, and/or adapting the antigen of the invention or an analogue thereof to a specific use, e.g. as a component in the vaccine such as described herein.

Also, a polypeptide part of the antigen of the invention may be prepared by the well-known methods of liquid or solid phase peptide synthesis utilizing the successive coupling of the individual amino acids of the polypeptide sequence or the coupling of individual amino acids forming fragments of the polypeptide sequence which fragments subsequently are coupled so as to result in the polypeptide part of antigen of the invention. The solid phase peptide synthesis may e.g. be performed as described by Merrifield RB (1963) p. 2149. In solid phase synthesis, the amino acid sequence is constructed by coupling an initial amino acid to a solid support and then sequentially adding the other amino acids in the sequence by peptide bonding until the desired length has been obtained. In this embodiment, the solid support may also serve as the carrier for the polypeptide part of the antigen of the invention in a vaccine preparation as described below. The preparation of synthetic peptides for use as vaccines or for diagnostic purposes may be carried out essentially as described in Shinnick, (1983), pp. 425-446.

Another aspect of the invention is a monoclonal or monospecific antibody reactive with *Plasmodium* species molecules, and a method for the preparation thereof. The term "antibody" refers to a substance which is formed by an animal or animal cell belonging to the immune system as a response to exposure to an antigen of the invention or an analogue thereof.

The antibodies of the present invention may be produced by a method which comprises administering in an immunogenic form at least a part of an antigen of the invention or an analogue thereof to obtain cells

producing antibodies reactive with said antigen or an analogue thereof and isolating the antibody containing material from the organism or the cells. The methods of producing antibodies of the invention will be explained further below.

The antibody is preferably a monospecific antibody. The monospecific antibody may be prepared by injecting a suitable animal with a substantially pure preparation of the antigen of the invention or an analogue thereof followed by one or more booster injections at suitable intervals (e.g. one or two weeks to a month) up to four or five months before the first bleeding. The established immunization schedule is continued, and the animals are bled about one week after each booster immunization, and antibody is isolated from the serum in a suitable manner (cf. e.g. Harboe and Ingild (1973) pp. 161-164.)

In another preferred embodiment, monoclonal antibodies are obtained. The monoclonal antibody may be raised against or directed substantially against an essential component of *Plasmodium* species molecules, i.e. an epitope. The monoclonal antibody may be produced by conventional techniques (e.g. as described by Köhler and Milstein, (1975) p. 495) e.g. by use of a hybridoma cell line, or by clones or subclones thereof, or by cells carrying genetic information from the hybridoma cell line coding for said monoclonal antibody. The monoclonal antibody may be produced by fusing cells producing the monoclonal antibody with cells of a suitable cell line, and selecting and cloning the resulting hybridoma cells producing said monoclonal antibody. Alternatively, the monoclonal antibody may be produced by immortalizing an unfused cell line producing said monoclonal antibody, subsequently growing the cells in a suitable medium to produce said antibody, and harvesting the monoclonal antibody from the growth medium.

The immunized animal used for the preparation of antibodies of the invention is preferably selected from the group consisting of rabbit, monkey, sheep, goat, mouse, rat, pig, horse and, humans and guinea pigs. The cells producing the antibodies of the invention may be spleen cells or lymph cells, e.g. peripheric lymphocytes.

When hybridoma cells are used in the production of antibodies of the invention, these may be grown *in vitro* or in a body cavity of an animal. The antibody-producing cell is injected into an animal such as a mouse resulting in the formation of an ascites tumour which releases high concentrations of the antibody in the ascites of the animal. Although the animals will also produce normal antibodies, these will only amount to a minor percentage of the monoclonal antibodies which may be purified from ascites by standard purification procedures such as centrifugation, filtration, precipitation, chromatography or a combination thereof.

An example of a suitable manner in which the monoclonal antibody may be produced is as a result of fusing spleen cells from immunized mice (such as Balb/c mice) with myeloma cells using conventional techniques (e.g. as described by Dalchau R et al. (1980), pp. 737-744). The fusions obtained are screened by conventional techniques such as binding assays employing *Plasmodium* species molecules isolated by the above-described methods.

In a further aspect, the invention relates to a diagnostic agent which comprises an antibody as defined above, preferably a monoclonal antibody. Alternatively, the diagnostic agent may be in the form of a test kit comprising in a container an antigen of the invention or an analogue thereof. The diagnostic agent may be used in the diagnosis of plasmodial infection. The diagnostic agent may be used to detect the presence of the plasmodial parasite or of a molecule related thereto in a sample as defined herein.

The diagnostic agent may be one which is suited for use in an agglutination assay in which the solid particles to which the antibody is coupled agglutinate in the presence of an antigen of the invention or an analogue thereof in the sample subjected to testing. In this type of testing, no labelling of the antibody is necessary. For most uses it is, however, preferred that the antibody is provided with a label for the detection of bound antibody or, alternatively (such as in a double antibody assay), a combination of labelled and unlabelled antibody may be employed. The substance used as label may be selected from any substance which is in itself detectable or which may be

reacted with another substance to produce a detectable product. Thus, the label may be selected from radioactive isotopes, enzymes, chromophores, fluorescent or chemiluminescent substances, and complexing agents.

Examples of enzymes useful as labels are β-galactosidase, urease, glucose oxidase, carbonic anhydrase, peroxidases (e.g. horseradish peroxidase), phosphatases (e.g. alkaline or acid phosphatase), glucose-6-phosphate dehydrogenase and ribonuclease.

Enzymes are not in themselves detectable, but must be combined with a substrate to catalyze a reaction the end product of which is detectable. Thus, a substrate may be added to the reaction mixture resulting in a coloured, fluorescent or chemiluminescent product or in a colour change or in a change in the intensity of the colour, fluorescence or chemiluminescence. Examples of substrates which are useful in the present method as substrates for the enzymes mentioned above are H_2O_2, p-nitrophenylphosphate, lactose, urea, β-D-glucose, CO_2, RNA, starch, or malate. The substrate may be combined with, e.g. a chromophore which is either a donor or acceptor.

Fluorescent substances which may be used as labels for the detection of the components as used according to the of invention may be 4-methylumbelliferyl-phosphate, 4-methylumbelliferylD-galactopyranoside, and 3-(p-hydroxyphenyl) propionic acid. These substances may be detected by means of a fluorescence spectrophotometer. Chemiluminescent substances which may be peroxidase/eosin/EDTA, isoluminol/EDTA/H_2O_2 and a substrate therefor.

Chromophores may be o-phenylenediamine or similar compounds. These substances may be detected by means of a spectrophotometer. Radioactive isotopes may be any detectable and in a laboratory acceptable isotope, e.g. ^{125}I, ^{131}I, ^{3}H, ^{35}P, ^{35}S or ^{14}C. The radioactivity may be measured in a γ-counter or a scintillation counter or by radioautography followed by densitometry.

Complexing agents may be Protein A, Protein G (which forms a complex with immunoglobulins), biotin (which forms a complex with avidin and

streptavidin), and lectin (which forms a complex with carbohydrate determinants, e.g. receptors). In this case, the complex is not in itself directly detectable, necessitating labelling of the substance with which the complexing agent forms a complex. The marking may be performed with any of the labelling substances described above.

In an embodiment of the invention an antibody or an antigen of the invention or an analogue thereof may be coupled to a bridging molecule coupled to a solid support. The bridging molecule, which is designed to link the solid support and the antibody, antigen or analogue may be hydrazide, Protein A, glutaraldehyde, carbodiimide, or lysine.

The solid support employed is e.g. a polymer or it may be a matrix coated with a polymer. The matrix may be of any suitable solid material, e.g. glass, paper or plastic. The polymer may be a plastic, cellulose such as specially treated paper. nitrocellulose paper or cyanogenbromide-activated paper. Examples of suitable plastics are latex, a polystyrene, polyvinylchloride, polyurethane, polyacrylamide, polyvinylacetate and any suitable copolymer thereof. Examples of silicone polymers include siloxane.

The solid support may be in the form of a tray, a plate such as a mitrotiter plate, e.g. a thin layer or, preferably, strip, film, threads, solid particles such as beads, including Protein A-coated bacteria, or paper.

The antigen of the invention or an analogue thereof and antibody of the invention may be used in an assay for the identification and/or quantification of at least a form and/or a part of the antigen or an analogue thereof present in a sample. The identification and/or quantification performed by the use according to the present invention may be any identification and/or quantification involving molecules of a *Plasmodium* species or a form thereof. Thus, both a qualitative and a quantitative determination of molecules of a *Plasmodium* species may be obtained according to the use of the present invention. The identification and/or quantification may be performed for both a scientific, a clinical and an industrial purpose. As will be

further described below, it is especially important in clinical routine to identify or quantify *Plasmodium* species molecules.

The sample may be a specimen obtained from a living organism such as a human or an animal, or an environmental specimen such as water. The specimen may be blood, e.g. an erythrocyte enriched fraction, or a tissue sample e.g. comprising liver cells. In a interesting embodiment of the present invention, the specimen is urine.

The identification and/or quantification may serve the purpose of diagnosing an infection with a *Plasmodium* species in an organism, e.g. an animal or an human being. The diagnosis is preferably performed on a specimen or sample from the organism, e.g. a specimen or sample of the type mentioned above. The identification and/or quantification may be performed by use of an assay in which the antigen of the invention or an analogue thereof or the antibody of the invention is employed. The antigen, analogue or antibody may be part of an assay kit of a composition suitable for its intended use. Such assay kits may comprise one or several layers and contain *Plasmodium* species molecules prepared by any of the methods described herein. This will be explained in further details below.

A drawback of some of the known methods for diagnosing malaria using clinical samples has been that known tests, when performed on samples of body fluids, principally whole blood, have not shown the specificity and sensitivity required for accurate diagnosis, and the one specific test, namely detection of the parasites in smears of peripheral blood obtained from an infected individual requires specially trained personnel, i.e. it cannot be performed as a routine analysis. Also it is unsuited as a screening analysis for the screening of a large number of patients.

The identification and/or quantification of *Plasmodium* species molecules in accordance with the present invention may be advantageous in accurate detection of e.g. recently acquired infection with *Plasmodium* species as readily available samples, in particular whole blood, plasma, serum or urine, may be used.

The malaria infection may be diagnosed by examining a sample, e.g. a blood sample, for the presence of antibodies against *Plasmodium* species molecules, the presence of *Plasmodium* species molecules, and/or the presence of a DNA or RNA fragment encoding the *Plasmodium* species molecules. Also, the presence and amount of *Plasmodium* species molecules in a vaccine, e.g. of the type disclosed herein, may be determined in this manner. As mentioned above, one aspect of the invention is the diagnosis of malaria infection performed on an urine sample. The use of an urine sample in the diagnosis of malaria is an easy and convenient approach for the diagnosis as compared to the use of a blood or serum sample.

In one preferred embodiment of the invention it is preferred that the antibody used in the method of the invention is a monoclonal antibody as this generally provides a higher precision and accuracy of the assay. Furthermore, a mixture of two or more different monoclonal antibodies may be employed as this may increase the detection limit and sensitivity of the test. The monoclonal antibody may be obtained by the method described above. Antibodies possessing high avidity may be selected for catching techniques.

The antibody used in the present method is preferably in substantially pure form (purified according to suitable techniques or by the methods of the invention, see below) in order to improve the precision and/or accuracy of the assays of the invention.

When the antigen of the invention or an analogue thereof or antibody of the invention is to be used for identification and/or quantification of *Plasmodium* species molecules it may be advantageous that the antigen of the invention or an analogue thereof or antibody is provided with a detectable marker or label. The detectable marker may be any marker which may easily be identified by means of conventional techniques and equipment, such as a radioactively labelled marker, e.g. an isotope such as ^{125}I (Doring G et al,.(1982) pp. 231-147), or an enzyme-labelled marker (Fitzgeralt DJ et al. (1984), pp. 966-971) or a marker labelled with fluorescein (Hoxic JA et al. pp. 1123-1127). Also a complexing agent such as biotin may be a useful marker.

The determination of antibodies reactive with the antigen of the invention or an analogue thereof of the invention and being present in a sample, e.g. as defined above, may be carried out by use of a method comprising contacting the sample with the antigen of the invention or an analogue thereof of the invention and detecting the presence of bound antibody resulting from said contacting and correlating the result with a reference value.

In one embodiment, the method of the invention employs some of the well known ELISA principles, e.g. direct (see Example 3), catching (see Example 12), competitive (see Example 5) and double enzyme linked immunosorbent. In e.g. an inhibition assay a purified antigen of the invention or an analogue thereof preparation of the invention is attached to a solid support (e.g. a polystyrene microtitre tray (Nunc); the test solution to be measured is mixed with specific reference antibodies, e.g. the antibodies of the present invention, and this mixture is incubated with the solid support provided with the antigen of the invention or an analogue thereof preparation as mentioned above. After sufficient washing, enzyme-labelled anti-IgG-antibodies are added, and finally enzyme substrate is applied, see Example 5. For further detailed information of the principles employed in ELISA techniques, cf. for instance Voller A et al. (1979).

The ELISA and RIA methods are well established and may be carried out with existing laboratory equipment and may also be subjected to automation. The methods of the inventions therefore has wide applicability in clinical laboratories for diagnostic purposes and for monitoring the results of vaccination procedures, and in the pharmaceutical industry as an assay for immunogens to be used in the production of vaccines.

When the antigen of the invention or an analogue thereof is to be employed in an assay for determining the presence of *Plasmodium* species molecules in a sample, it may be in the form of a diagnostic reagent or a diagnostic agent. As will be apparent to a person skilled in the art several techniques may be applied in connection with such diagnostic reagents.

As stated above, infection by *Plasmodium* species molecules in an organism or the presence of such in a sample may be detected by determining the presence of a DNA sequence related to the *Plasmodium* species molecules using a DNA sequence encoding an antigen of the invention or an analogue thereof. The detection is based on homology between DNA sequences in the sample and the DNA sequence of the invention and may be performed by use of a diagnostic agent comprising a labelled DNA sequence homologous with a DNA sequence encoding at least part of the antigen of the invention or an analogue thereof of the invention. The DNA sequence may be labelled with any suitable label, e.g. selected from radioactive isotopes, enzymes, chemical modifying agents such as sulphonyl-introducing compounds and complexing agents such as biotin.

The use of a DNA fragment for the detection of the presence of DNA sequences related to *Plasmodium* species molecules in a sample may advantageously be carried out utilizing the principles of the polymerase chain reaction. The polymerase chain reaction (PCR) is a procedure used for the amplification of DNA present in a sample and is, for instance, described by Randall et al. (1985), Randall et al., (1988), and Stoflet et al. (1988).

The invention is further illustrated but not limited to the following examples and appended drawings. In the drawings,

Fig 1 shows the result of a crossed immunoelectrophoresis analysis of affinity purified antigen 7 tested against polyspecific human immune serum, i.e. as described in Example 1;.

Fig 2 the result of a crossed immunoelectrophoresis analysis of affinity purified antigen 7 incubated with a) NaCl, b) trypsin, c) α-D-galactosidase and d) phospholipase C and tested against a polyspecific human immune serum;

Fig 3 the result of a crossed immunoelectrophoresis analysis of affinity purified soluble antigens tested against polyspecific human immune serum with a) 10 $\mu l/cm^2$ inactivated LAL and b) 10 $\mu l/cm^2$ LAL

incorporated in the first dimension gel. The arrow indicate the disapperance of the antigen 7;

Fig 4 the result of a test of antigen 7 in immunoblotting against 1) control non-immune human serum and 2) human immune serum.

Fig 5 the immunoprecipitation pattern of synchronized parasited cultures.

the immunoprecipitation was performed with a) extracted parasites and b) concentrated culture supernatant (x5) tested against mouse anti anti antigen 7 antibodies.

Time intervals of the parasited cycle 1) 15-20 h, 2) 20-25 h, 3) 25-30 h, 4) 30-35 h, 5) 35-40 h, and 6) 40-45 h.

The 59 kDa component is abundant in the culture supernatant, but only weakly detectable in this picture.

Fig 6 shows the esult of a test illustrating interleukin-1 production (units/ml) of peripheral blood mononuclear cells after stimulation with A) medium B) affinity purified soluble antigens, C) affinity purified soluble antigens minus antigen 7, D) antigen 7, E) antigen 7 control buffer and f) PPD. Individual and mean response to each type of stimulation is shown;

Fig 7 whoes the result of a test of TNF production (units/ml) of peritoneal cells after stimulation with antigen 7 components separated by SDS-PAGE and immunoblotting;

Fig 8 shows a electron micrograph of antigen 7 incorporated in ISCOM structure.

Fig 9 shows a test of mouse-anti-antigen 7 serum in crossed immunoelectrophoresis (CIE). CIE of affinity purified soluble antigen tested against a polyspecific humane immune serum with a) 100 µl control mouse serum and b) 100 µl mouse anti antigen 7 (from mouse immunized with antigen 7 ISCOM's) incorporated in the intermediate gel;

Fig 10 shows the proliferative response of peripheral blood mononuclear cells to affinity purified soluble antigens and antigen 7 after 7 days in culture (Δcpm). Each point represents the mean response in triplicate wells for one individual.

I = immune, NI = nonimmune.

Background kcpm of unstimulated cultures were in the range of 0.1 to 2.3 kcpm. There was no significant difference between immune and nonimmune donors in the activity of unstimulated cultures (Wilcoxin rank sum test. P>0.1);

Fig 11 shows the result of a pyrogenicity test with antigen 7. The temperature difference from the initial temperature is shown at 30 min. intervals for rabbits (mean of 3 rabbits ± 2 x standard error) injected i.v. with:

antigen 7 shown by _____
antigen 7 + rabbit anti antigen 7 Ig shown by - - - - ; and

Fig 12 shows the prevalence of precipitating antibodies directed against antigen 7 tested in crossed immunoelectrophoresis. Plasma from gambian donors of different age groups were tested.

----- denotes high reactivity with antigen 7
- - - denotes crossed reactivity with antigen 7;

Fig 13 shows the prevalence of precipitating antibodies directed against antigen 7 tested in crossed immunoelectrophoresis. Plasma from gambian children 3-8 years old were tested. Plasma samples from May (dry season) and November (wet season) were tested.

Group 0: no reactivity with antigen 7
Group 1: weak reactivity with antigen 7
Group 2 + 3: strong reactivity with antigen 7

The donors were divided into 3 categories.

Group 0: donors who showed no sign of malaria during the rainy season
Group 1: donors who had malaria during the rainy reason
Group 2 + 3: donors who had malaria parasites during the rainy season but who did not show any sign of disease.

MATERIALS AND METHODS

Parasite cultures

The *P. falciparum* isolate F32/Tanzania was kept in continuous culture in a modified Trager and Jensen system (see Trager and Jensen) (1976) as described by Jepsen et al. (1981). Leucocytes were removed from acid citrate dextrose samples of human A^+ blood by adsorption to a Whatman CF11 cellulose column (Whatman Ltd., Springfield Mill, Maidstone, Kent, UK). Parasites were grown in aliquots of 4 ml 10% erythrocyte suspensions kept in 50 ml Nunc culture flasks (Nunc, Roskilde, Denmark) and incubated at 37°C. Culture medium was RPMI 1640 supplemented with 21 mM sodium bicarbonate, 25 mM HEPES buffer and 6% human serum. Medium was changed every 24 hours and the collected supernatants were stored at -20°C. At weekly intervals when parasitemia (percent infected erythrocytes) had risen to 6-15%, the cultures were diluted to approx. 0.2% parasitemia with a 10% human Apositive erythrocyte suspension and distributed in 4 ml aliquots. For metabolic labelling experiments cultures were synchronized by a combination of sorbitol and aphidicolin treatment. Parasite cultures were centrifuged and 9 volumes of 5% (w/v) sorbitol in water was added to the pellet and mixed. After 10 min at 37°C, the mixture was centrifuged at 2000 rpm for 5 min. The pellet was then used for culture. 18 hours later, this sorbitol treatment was repeated and the parasites were grown in medium with 1 µg/ml aphidicolin (Sigma, St. Louis, USA) for 15 hrs; After further culture for 15 hours in normal medium, the cultures were centrifuged and the pellet mixed with 4 ml of "plasmagel" plus 3 ml medium and incubated at 37°C for 30 min. This synchronised culture was used for labelling studies.

Immune sera

Sera were obtained from malaria-immune adult blood donors and malaria exposed donors from Indonesia, Liberia, Gambia, Guinea Bissau, Sudan and Mozambique.

Saponin treated parasite antigens

Parasite cultures were centrifuged at 2400 x g for 5 min. Cell pellets were washed in 5 volumes sterile 0.9% NaCl, centrifuged at 2400 x g for 5 min and incubated in 5 volumes of a 0.01% saponin solution in sterile water for 10 min at room temperature. After centrifugation at 4500 x g for 10 min, the saponin treatment was repeated and the pellets were finally washed in 5 volumes of sterile isotonic NaCl and centrifuged at 800 x g for 10 min. The cell pellet was discarded and the supernatant was used for SDS-PAGE.

Extraction of antigens from parasite-infected erythrocytes

1 volume pelleted parasite-infected erythrocytes was extracted with 7 volumes of 50 mM TRIS-HCL pH 7.4, 1% Triton X-100, 100mM NaCl, 1mM EDTA, 0.25% NP40, 0.02% NaN_3, 0.25% BSA at 0°C for 30 min. The mixture was then centrifuged at 5600 x g for 30 min. and the supernatant was collected for use in immunoprecipitation studies.

Immunosorbent techniques.

Materials:

Pump:	Varioperpex 1, LKB, Stockholm, Sweden.
Recorder:	LKB, Stockholm, Sweden, model 2210.
UV-monitor:	Pharmacia Fine Chemicals, Uppsala, Sweden, model UV-1, 280 nm.
Concentration cell:	Amicon cell, model 202, ultrafilter PM 10 (Amicon Corp., Lexington, MA, USA).
Filter:	0,22 um from Schleicher and Schuel.
Fraction collector:	Redirac, LKB, Stockholm, Sweden.

Buffers:
Column buffer: 0,02 M Tris barbital pH 8.6, 0.5 M sodium chloride, 15 mM NaN3.

Elution buffer: 3 M potassium thiocyanate in column buffer.

Limulus amoebocyte lysate (LAL) test.

All reagents used in the preparations of the malaria antigens were tested for their endotoxic activity by a combination of the LAL test with rocket immunoelectrophoresis as described in Baek L (1983), using LAL from Cape Cod (Association of Cape Cod, Inc., Woods Hole, MA, USA). The sensitivity of LAL was 1 pg/ml measured by *Escherichia coli* 055:B5 standard endotoxin (Mallinckrodt, Inc., St Louis, MO, USA).

EXAMPLE 1

ISOLATION AND CHARACTERIZATION OF ANTIGEN 7

Isolation of soluble P. falciparum antigens using affinity chromatography

Supernatants from the daily change of exhausted medium from parasite cultures were pooled, dialysed against column buffer, (TRIS barbital pH 8,6, 0,5 M NaCl, 15 mM NaN_3) and added to a CNBr-sepharose 4B column (260 ml gel)(Pharmacia Fine Chemicals, Uppsala, Sweden) containing as ligand a pool of IgG (2.5 mg/ml gel) from Liberian adults clinically immune to malaria and with high titres of precipitating antibodies against soluble *P. falciparum* antigens. Bound antigens were eluted with 3M potassium thiocyanate in 0,02M TRIS-Veronal buffer pH 9,0. The pooled fractions containing eluted antigens were

dialysed, concentrated x 250 and tested for their content of soluble antigens in CIE as described below.

Isolation of antigen 7

100 mg IgG from a Liberian donor, with precipitating antibodies only reacting with antigen 7 when tested in CIE, e.g. as described below, was purified by protein A affinity chromatography and coupled to 50 ml divinyl sulfone agarose (2 mg/ml gel) (Kem En Tec, Denmark). 3 ml of affinity purified soluble antigens obtained as explained above were added to the column. After washing with column buffer (TRIS barbital pH 8,6, 0,5 M NaCl, 15 mM NaN_3), bound antigen 7 was eluted with 3 M potassium thiocyanate in 0,02M TRIS-Veronal buffer pH 9,0, dialyzed against column buffer and concentrated 50 times.

Crossed immunoelectrophoresis (CIE)

CIE was performed on glass plates 7 x 5 cm in 1% agarose gel (Litex, Glostrup, Denmark, type HSA) in Tris-barbitol buffer pH 8.6 ionic strength 0.02 by running 20 µl of the affinity purified soluble antigens described above or 20 µl of affinity purified antigen 7 in the first dimension gel at 10-15 Volt/cm until a parallel blue albumin marker had migrated 2,6 cm. The second dimension electrophoresis was run perpendicular to the first dimension gel at 2 Volt/cm for 18 hours into a gel containing 16 $\mu l/cm^2$ human Liberian immune serum as defined above. The plates were washed and pressed three times and stained with Coomassie brilliant blue R250. The results are shown in Fig. 1. Only antigen 7 is seen in the column eluate. Antigen 7 may occur in two parallel precipitates.

Enzymatic digestion

Enzyme digestion was performed with the following enzymes: α-D-galactosidase (Sigma) 0,2 units added to 20 µl antigen 7; incubated at room-temperature overnight.

Trypsin (Sigma), 200 μg added to 20 μl antigen 7 with 10 mM $CaCl_2$; incubated at room temperature for 30 min.

Phospholipase C (Sigma) from *Bacillus cereus* (heated for 10 min before use) 40 units added to 20 μl of antigen 7 with 10 mM CaCl, 1 mM β-mercaptoethanol, 0,02% Triton X-100, incubated at 37°C for 1 hour.

Phospholipase A (Sigma) from bee venom (heated for 10 min before use) 40 units added to 20 μl antigen 7 with 0,1% Na-deoxycholate, 1 mM NaCl, 0,1 M Tris-HCl pH 7,4 incubated at 37°C for 1 hour.
Neuraminidase (Sigma), 1 unit added to 20 μl antigen 7 and incubated at 37°C for 30 min.

Treatment with phospholipase C and trypsin destroyed the antigenicity of antigen 7 in CIE, while treatment with α-D-galactosidase altered the position of antigen 7 (Fig. 2). Neuraminidase or phospholipase A_2 treatment had no effect (data not shown).

Preparations of parasite antigens with LAL in CIE.

To study the reaction of LAL with different parasite antigens, purified soluble antigens were mixed with LAL in test tubes in the ratio of 1:2 (v/v) and incubated at 37°C for 60 min. Antigens diluted 1:2 (v/v) with pyrogen-free 0.154 M NaCl and incubated under identical conditions served as control. The antigen-LAL mixture was applied to the wells of the electrophoresis gel immediately after the incubation period, and electrophoresis was performed. CIE was also performed by incorporating 10-20 $\mu l/cm^2$ LAL either in the first dimension gel or in the intermediate gel. To avoid denaturation of proteins, LAL was added to the melted gel just before it was poured onto the glass plate. As a control, 0,154 M NaCl was used instead of LAL in the same volumes as mentioned above. A blank intermediate gel was interposed between the LAL-containing and the antibody-containing gel to increase resolution. In another control experiment, LAL was heated to 60°C for 30 min to inactivate the enzymes in LAL before incubation with the parasite antigens. Incubation of the antigens with LAL

eliminated precipitate no. 7. Incorporation of LAL in the first dimension gel (Fig 3) or in the intermediate gel also eliminated precipitate no. 7.

Immunoblotting

675 µl affinity-purified antigen 7 were heated at 56°C for 10 min and applied to an SDS-PAGE slab gel consisting of 5% stacking gel and 12% separation gel. Marker proteins (BIO RAD, Richmond, CA, USA) were: Myosin (200 kDa), Beta-galactosidase (116.25 kDa), Phosphorylase C (92.6 kDa), Bovine Serum Albumin (66.2 kDa), Ovalbumin (45 kDa), Carbonic anhydrase (31 kDa), Soybean Trypsin inhibitor (21.5 kDa), and Lysozyme (14.4 kDa). Electrophoresis was performed at 25-40 Volt for 17 hours under reducing conditions with 100mM dithiothreitol (Sigma) in the sample buffer. The antigens were then electro-transferred to nitrocellulose sheets in a semi-dry electroblotter (ANCOS, Ølstykke, Denmark). The nitrocellulose sheets were blocked with TRIS-HCL pH 7.4, 0.5% (w/v) BSA, 0.5% (v/v) Tween 20 and incubated for 1 hour with Liberian immune sera as defined above diluted in a washing buffer, TRIS-HCL pH 7.4, 0.05% Tween 20. After washing, the sheets were incubated with peroxidase-conjugated rabbit anti-human Ig, rabbit anti-mouse IgG or swine anti-rabbit IgG (Dakopatts, Copenhagen, Denmark) diluted 1:2000 in washing buffer. The sheets were then washed and stained with 15 ml DONS/TMB [120 mg Dioctyl sodium sulfosuccinate (DONS) + 3,6 mg Tetra methyl benzidine (TMB) + 15 ml ethanol (96%)] + 45 ml citrate phosphate pH 5,0 + 30 µl H_2O_2 (30%). The results are shown on Fig. 4. Antigen 7 is present as 3 bands of 77, 61 and 59 kDa. Sometimes low molecular mass bands are also present.

Immunoprecipitation of synchronized cultures

Immunoprecipitation was performed with Triton X-100 extracted parasites (see Materials and Methods) and concentrated culture supernatant (x5) volume from ^{35}S-methionine labelled cultures. Samples were taken at five hour intervals beginning 15 h after the start of the parasite

cycle. 100 µl parasite antigen (1 x 10^6 cpm) were preabsorbed to Protein A sepharose for 30 min and centrifuged. The supernatant was added to 14 µl mouse anti-antigen 7 antibodies (purified by Protein A sepharose chromatography) and incubated over night at 4°C. Then 50 µl Protein A sepharose (Pharmacia) was added, incubated at room temperature for 1 hour and centrifuged at 2400 x g for 5 min at 4°C. 3 washes were performed with 0.01 M TRIS-HCL pH 7.8, 2% Triton X 100 (v/v), 0.6 M KCL, 0.15M NaCl, 0.005 M EDTA, followed by 1 wash with PBS. The samples were heated at 56°C for 10 min before SDS-PAGE was performed under reducing conditions with (DTT). Autoradiographs were exposed for 3 weeks at -80°C. The immunoprecipitation pattern obtained is shown in Fig. 5. It appears that antigen 7 is first detected as a 130 kDa antigen precursor in early schizonts (35 hours) and that 77 and 59 kDa components become dominant later (40 hours) in schizont development (Fig. 5). Only the 77 and 59 kDa components are released into the culture medium at schizont rupture.

EXAMPLE 2

CYTOKINE STIMULATION

Interleukin-1 production

To test the antigens ability to induce IL-1 production, mononuclear cells (MNC) from Danish donors were stimulated by affinity purified soluble antigens, affinity purified antigen 7, affinity purified soluble antigens excluding antigen 7, antigen 7 buffer control (buffer processed as antigen 7) and Pufified Protein Derivative of tuberculin (PPD) (State Seruminstitute, Copenhagen, Denmark). In some experiments the antigens were boiled at 100°C for 30 min before assay. The malaria antigens and the buffer control were tested at 4 dilutions. Antigens and 1 x 10^5 MNC were co-incubated in roundbottomed microtitre plates in a final volume of 100 µl RPMI 1640 containing 15% control human serum at 37°C in 5% CO_2. After 18 hrs, the supernatants were collected and stored at -20°C until IL-1 assay. IL-1 production was measured by bioassay using the murine thymoma EL4 cell line (16). In the presence of IL-1 and the calcium ionophore

A23187 (Sigma), EL4 cells produce interleukin-2 (IL-2). Serial dilutions of test supernatants were incubated with 2 x 10^5 EL4 cells/200 µl culture in the presence of 2.5 x 10^{-7} M A23187 for 18 hrs at 37°C, and 50 µl of the supernatants were collected and tested for IL-2, determined by a modification of the bioassay described by Gillis et al (1987).

It was shown that antigen 7 induced production of IL-1 in MNC cultures, and that this production was higher than in cultures stimulated by control antigen 7 buffer (Fig. 6). Affinity purified soluble antigens depleted of antigen 7 was less efficient in inducing IL-1 production than affinity purified soluble soluble antigens. In order to test whether lipopolysaccharide (LPS) contamination of the antigen 7 preparation was responsible for the induction of IL-1, antigen 7 was boiled before addition to the cultures. It was shown that boiling diminished the IL-1 production in antigen 7 cultures to the levels of buffer stimulated cultures, whereas the production in cultures stimulated with boiled PPD was unaffected.

Table 1

Stimulation of IL 1 production (units/ml) from mononuclear cells by boiled and unboiled preparations of Ag7, Ag7 controlbuffer and PPD. Median and range of three experiments is shown.

	No boiling	Boiling
No stimulation	0 (0-0)	20 (0-40)
Ag7	120 (75-300)	40 (20-50)
Ag7 controlbuffer	50 (19-50)	50 (0-75)
PPD	300 (200-400)	200 (75-600)

Stimulation of Tumour Necrosis Factor (TNF) in vitro by antigen 7

a) Peritoneal cells

Peritoneal cells were collected from mice which 3-5 days previously had been given 1 ml of 4% thioglycollate (Difco) i.p., using Hank's buffered saline solution (BSS) (Flow Laboratories) containing 1 U/,l of heparin and 5 µg/ml of polymyxin B (Sigma). Washed cells were suspended in 5% fetal calf serum (FCS) in RPMI 1640 containing polymyxin B, counted under a microscope using acridine orange and ethidium bromide for staining of the cells, adjusted to approx. 1 x 10^7 viable cells per ml, and then 0.1 ml volumes were dispensed into wells of 96-well microtitre plates (Nunc, Roskilde, Denmark). The cells were incubated for 2-3 hours at 37°C in an atmosphere of 5%

CO_2 in air to allow macrophages to adhere, and then for a further 30 min with an equal volume of medium containing 2 μg/ml indomethacin (Sigma). Non-adherent cells were washed away with BSS free of polymyxin B, the medium was replaced by 0.2 ml volumes of RPMI 1640 containing dilutions of antigen 7 to be tested, and the cultures were incubated overnight. On the next day, supernatants were collected and assayed for TNF. A 1/10 dilution of each was made in medium containing 5% FCS and 1 μg/ml of emetine (Sigma), and stored at -20°C in case a titration needed to be repeated. Cultures incubated with LPS or with medium alone were included in every experiment as positive and negative controls on the capacity of the macrophages to yield TNF.

b) Human monocyte cultures

60 ml of blood from unexposed donors was defibrinated by shaking with glass beads in a glass universal and the mononuclear cells separated by centrifugation over lymphocyte separation medium (Flow Laboratories). The cells were washed and suspended in RPMI 1640 supplemented with 10% autologous serum and cultured overnight in a flask which had been prewashed with undiluted autologous serum. Next day non-adherent cells were washed off with three rinses of RPMI 1640 and the monocyte-enriched population was detached, by incubating at 37°C with RPMI 1640 containing 10% autologous serum and 3.3. mg/ml of disodium EDTA. After 30 min, the cells were removed by gentle pipetting, centrifuged once and these cells were suspended in RPMI 1640 and dispensed in flat-bottomed 96-well microtitre plates at concentrations of 1 x 10^5 cells/well, when TNF yields were to be assayed by ELISA as described below, or at 5-10 x 10^5 cells/well, when they were to be assayed for cytotoxicity as described below. The cells were pretreated with recombinant human gamma interferon (100 U/ml) (Genentech) and indomethacin (1 μg/ml) for 3 hours, and then incubated overnight with serial dilutions, made in RPMI 1640, of antigen 7 to be tested. Next day, supernatants were collected and either stored at 4°C (for ELISA assay) or treated like peritoneal cell supernatants (for the bioassay).

ASSAYS ILLUSTRATING THE PRESENCE OF TNF

1) Cytotoxicity assay for TNF

All samples containing murine TNF, and some containing human TNF, were assayed colorimetrically by their cytotoxicity for L929 mouse fibroblast cells.

Flat-bottomed microtitre wells were seeded with 1.5 x 10^4 L929 cells in 0.1 ml RPMI 1640 supplemented with 10% heat inactivated horse serum and 5 µg/ml actinomycin D (Sigma) and 1 µg/ml emetine. The cells were exposed to serial dilutions of antigen 7 in RPMI 1640 medium. After 48 hours of incubation, the cells were fixed with formalin and stained with crystal violet.

The microtitre plates were dried after staining and the absorbance was determined for each well in a Titertek Multiscan automatic microtiter plate reader. Each dilution of antigen 7 was tested in duplicate. Control values were based on the mean absorbancy at 620 nm of L929 cells cultured without antigen. One unit is defined as that causing 50% cell destruction.

For assays of cytotoxic activity in supernatants from human monocytes, L929 cell monolayers were incubated with 100 U/ml of gamma interferon for 24 hr, this enhances the sensitivity to human TNF about 50-fold.

2) ELISA assay for human TNF

A monoclonal antibody specific for TNF was used for coating the wells. After coating and blocking the wells, the supernatants to be assayed were added. After wash a biotinylated monoclonal antibody to TNF was added. After wash binding of the biotinylated antibody was detected with a streptavidin-peroxidase complex (RPN-1051, Amersham International, U.K.) used at 1/500 in PBS containing 2% bovine serum

albumin and incubated for 30 min at 37°C. Substrate was 2,2-Azino di-3-ethyl benzothiazoline sulphonic acid and H_2O_2 used according to standard protocols. To convert absorbance values into units, a series of dilutions of a standard preparation of recombinant human TNF were run in parallel with monocyte supernatants (National Institute for Biological Standard and Control, Poters Bar, U.K.).

3) Antiserum against parasite soluble antigens

Groups of mice were injected i.p. with 0.2 ml of boiled *P. falciparum* culture supernatant and were bleed 7-12 days later. Each pool of antiserum was checked to confirm that it inhibited the ability of the parasite antigens, but not of LPS, to induce the release of TNF by mouse macrophages.

The results obtained with mouse peritoneal cells incubated with antigen 7 is shown in Table 2. Antigen 7 stimulates the production of TNF over a broad dilution range. Furthermore, Table 2 shows that this TNF stimulation can be blocked by mouse antiserum to soluble *P. falciparum* antigens, indicating that antibodies block the capacity of antigen 7 to stimulate TNF production *in vitro*.

Table 2

Inhibition of TNF stimulation by antigen 7 by mouse antiserum against P. falciparum boiled supernatant

Antiserum: taken from mouse day 8 after 0.2 ml antigen diluted 1/5 given i.p.

Trigger	Diln	TNF U/well	AB diln	TNF/U well
Ag7	1/20	13,511	1/50	0
	1/40	8,914		0
	1/80	6,755		0
	1/160	4,457		0

To localize the fragment of antigen 7 responsible for the TNF stimulation, affinity purified antigen 7 was subjected to SDS-PAGE and immunoblotting onto Schleischer and Schnell nitrocellulose sheets. Different molecular mass intervals of antigen 7 was tested by cutting the sheets into 0.5 x 1.0 cm pieces. Each piece was dissolved in 0.2 ml methanol and evaporated. Testing was performed in cell cultures (10 cells/well) preactivated with gamma interferon and indomethacin. Results are shown in Fig. 7. The 16 kDa fraction possessed the strongest TNF stimulating capacity.

EXAMPLE 3

IMMUNIZATION

Quil A-antigen 7 preparation

Affinity purified antigen 7 (10 volumes) was mixed with 15 mg/ml Quil A (Lindholm, Denmark) (1 volume) and dialyzed against PBS (pH 7,2) for 4-6 hours at room temperature and then overnight at 4°C. The antigen 7 Quil A mixture was shown to form an immune stimulating

complex (ISCOM) Structure (Morein B, 1984) when tested by electron microscopy (Fig. 8)

A) Immunization of mice and rabbits with purified antigen 7

Conventional rabbits (Ssc:CPH), aged approx. 3 months, were immunized subcutaneously four times at weekly intervals with 200 µl affinity purified antigen 7 in 200 µl of Freund's complete adjuvant (FCA). The rabbit sera were used for *in vitro* inhibition experiments.

Conventional female mice (BALB/cJ/Ssc), at least 2 months old, were immunized subcutaneously four times at weekly intervals either with 100 µl of affinity purified antigen 7 in 100 µl Freund's complete adjuvant, or with 50 µl affinity purified antigen 7 in ISCOM's for immunogenicity and antigen characterization studies.

Mouse and rabbit sera were tested for antibody reactivity by incorporating 15 $\mu l/cm^2$ mouse serum or 40 $\mu l/cm^2$ rabbit serum in the intermediate gel of a CIE. A blank intermediate gel was interposed between the two antibody-containing gels to increase resolution.

It was seen that immunisation of mice with antigen 7 ISCOMs and immunization of mice and rabbits with antigen 7 stimulated production of precipitating antibodies against antigen 7 which could be detected in CIE (Fig. 9).

Other antigen 7 preparations and/or immunization procedures may be used, e.g. in accordance with the procedures outlined below.

B) Presentation of antigen 7 in ISCOM's purified by ultracentrifugation for immunization

A mixture of 900 µl affinity purified antigen 7 and 100 µl (15 mg/ml) Quil A is dialyzed against PBS under stirring at room temperature for 4-6 hours and then overnight at 4°C.

Subsequently, the antigen is applied onto a layer of 500 µl 5% sucrose plus 0.1% Triton X-100 in 0.05 M Tris buffer plus 0.1 M NaCl which was layered over a 2.5 ml 10% sucrose in 0.05 M Tris buffer plus 0.1 M NaCl. The centrifugation was performed in a Bechmann SW 50 rotor at 150.000 x g for 4 hours at 20°C.

The pellet is collected and dialyzed extensively against column buffer at 4°C. The abundance of ISCOM's is confirmed by electron-microscopy. The abundance of antigen 7 in the ISCOM's is confirmed by immunoblotting. The antigen 7-ISCOM structure is used for immunization studies in BALB/c mice as described in section A above.

C) Immunization with antigen 7 separated by SDS-PAGE

Immunoblotting as described in Example 1. Antigen 7 bands are cut out of the nitrocellulose sheath in a size of about 0.5 x 10.0 cm. The nitrocellulose sheeths are then cut into pieces and dissolved in 1 ml dimethylsulphoxid or methanol. The dissolved bands are to be used for immunization following the schedule described in section A above. Rabbits are immunized with 250 µl dissolved band plus 250 µl Freunds complete adjuvans three times with two week intervals. Mice are immunized with 100 µl dissolved band plus 100 µl Freunds complete adjuvans three times with two week intervals.

The dissolved bands are used for lymphocyte proliferation studies in serial dilutions of 1:10-1:250. 20 µl of each dilution is added to each well and the assay is performed as described in Example 5.

EXAMPLE 4

Cloning of the gene encoding antigen 7

P. falciparum culturing was performed as described above in Materials and Methods, parasite cultures. DNA was extracted from the parasites of the schizont stage present in the red blood cells according to the following procedure:

1. The red blood cells were sedimented by gravity.
2. 2/3 of the medium was removed carefully so as not to stir the red blood cells.
3. The suspension was centrifuged at 2000 x g_{av} for 5 min and the supernatant was removed.
4. The cell pellet was washed in a volume of isotonic saline constituting approx. 5 times that of the pellet and centrifuged at 2000 x g_{av} for 5 min.
5. The pellet was resuspended in 0.01% saponin in isotonic saline and incubated for 10 min at room temperature.
6. Centrifugation at 3000 x g_{av} for 10 min.
7. The pellet was resuspended in a volume of 0.01% saponin in isotonic saline constituting approx. 5 times the volume of the pellet and incubated at room temperature for 5 min followed by centrifugation at 3000 x g_{av} for 10 min.
8. The pellet was washed in a volume of isotonic saline constituting approx. 5 times the volume of the pellet and centrifuged at 3000 x g_{av} for 10 min.
9. The pellet was suspended in a volume of DNA buffer (100 mM Tris-HCl pH 8.0, 1% SDS, 50 mM EDTA, 0.2 M NaCl) constituting approx. 5 times the volume of the pellet.
10. RNase A which had been boiled for 10 min was added to 50 microgram/ml and the suspension was incubated at 37°C for 1 hour.
11. Proteinase K was added to 100 microgram/ml, and the suspension was incubated at 50°C for 1 hour.
12. Phenol extraction and ethanol precipitation were performed according to Maniatis et al., (1982), pp.458-459 and 461-462.
13. The pellet was redissolved in a suitable volume of 10mM Tris-HCl pH 7.5, 1mM EDTA.
14. The concentration of DNA was estimated by measurement of OD_{260} and OD_{280} according to Maniatis et al., (1982) p. 468.

5 ml of a solution of 100 microgram DNA/ml was placed in a syringe with a 25 Gx1 cannula, all was stored on ice for 1 hour. The DNA

solution was pressed rapidly out of the syringe so as to shear the DNA. This resulted in the formation of the DNA molecules having an average size of 20 kbp as measured by gel electrophoresis. Phenol extraction was performed as described above, and EDTA was added to a concentration of 25mM. An isopropanol precipitation was performed as described in Maniatiset al., (1982), pp. 461-462. DNA fragments were made blunt ended by filling out with T4 DNA polymerase in the presence of the four types of deoxynucleotides, as described in Maniatis et al., (1982) p. 117-121. EcoR1 linkers from New England Biolabs were ligated to the DNA fragments as described in Maniatis *op. cit.*, p. 243-246. The resulting DNA fragments with EcoR1 linkers were digested with EcoR1 (Boehringer-Mannheim) and ligated to lambda gt11 arms (Promega Biolabs). The ligations were performed with T4 DNA ligase (Amersham). The recombinant lambda genomes were packaged with a packaging mix (Promega biolabs) according to the instructions of the manufacturer. The library was then used to infect Y1090 and plated on LB medium with agar. Nitrocellulose blots (BA85 membranes, Schleicher und Schuel) of 94 plaques expressing malaria proteins were screened with serum from Liberian malaria immune adults to detect clones expressing malaria proteins (Young RA and RW Davis, 1983). The sera were selected for their monospecific reactivity with antigen 7 when tested in crossed immunoelectrophoresis against all the soluble *P. falciparum* antigens, the visualization was performed with a pig antihuman IgG antibody conjugated to horseradish peroxidase (DAKOPATT P214) followed by a peroxidase staining (Heegaard, NHH and OJ Bjerrum, 1987). Five clones were positive after two screening procedures and one clone φ19 was selected for further studies.

The antigen 7 insert of the positive clones, e.g. clone φ19, can be sequenced by use of restriction fragment mapping. The insert is digested with a suitable enzyme in order to produce fragments of a length suitable for sequencing, and the resulting fragments are sequenced using, e.g., the Sanger dideoxy termination sequencing technique using single and double-stranded DNA (cf. Sanger, F.; 1981, and United States Biochemical, 1988)

Expression of the DNA sequence encoding antigen 7 or an analogue thereof by infectious recombinant vaccinia virus.

The DNA sequence encoding antigen 7 or an analogue thereof may be inserted into the vaccinia virus genome under the control of a defined vaccinia virus promotor. The expression can be facilitated by construction of plasmid insertion vectors which contain a defined vaccinia virus promotor upstream from unique restriction endonuclease sites and flanked by vaccinia virus DNA from a nonessential region of the virus genome. The DNA sequence encoding antigen 7 or an analogue thereof is placed under the control of the vaccinia virus promotor and inserted into infectious virus by homologeous recombination *in vivo*. By inserting the DNA sequence encoding antigen 7 or an analogue thereof into the thymidine kinase (TK) locus at the EcoRI site, recombinants display a selectable TK^- phenotype.

Virus recombinants are selected by loss of vaccinia virus TK expression in a plaque assay in human TK^- 143 cells in the presence of 25 µg/ml 5-bromodeoxy uridine.

For the production of recombinant protein CV-1 cell monolayers can be infected with purified wild-type or vaccinia virus recombinants at 30 plaque forming units per cell. At 2 hrs, virus inoculum are replaced with 2.5 ml of Eagle medium containing 2.5% fetal bovine serum. Cells are collected at 24 hours and separated from culture medium by centrifugation at 2,000 g for 5 min. Cell pellets are suspended in 2.5 ml of phosphate-buffered saline, freeze thawed three times and sonicated. Culture supernatants and cell extracts are tested for the presence of recombinant proteins.

Expression of antigen 7 or an analogue thereof from recombinant DNA clones in mammalian cells

Recombinant plasmids are incubated at 50°C in the presence of S1 nuclease. At this temperature, the poly (dA, dT) joints melt, and the resulting single strands are digested with nuclease S1. The fragments containing antigen 7 sequences are separated by electrophoresis and

after isolation, treated with DNA polymerase I to convert the ragged single-stranded ends to "blunt" ends. A synthetic decanucleotide is then attached to either end of the DNA with the aid of T4 DNA ligase. The resultant molecules are digested with endonuclease HindIII and cloned in the *E. coli* vector pBR322 to enrich the DNA. DNA is excised from recombinant pBR322 by sequential digestion with HindIII and Bgl endonucleases. The DNA fragment will then be ligated to SVGT-5 type of the SW-40 vector. To propagate the DNA production, monkey kidney cells are infected at 41°C with a mixture of SVGT-5 - antigen 7 DNA and the DNA from a tsA mutant of SW40, which provides late gene functions. Plaques are screened by plaque hybridization *in vitro*.

In a similar manner, antigen 7 or an analogue thereof may be produced in other mammalian cell line systems under conditions suited to the system in question.

EXAMPLE 5

Lymphocyte proliferation assay (Fig. 10)

Heparinised venous blood was collected from 33 adult Gambian donors who were clinically immune to malaria. Malaria transmission in the Gambia is intense and seasonal with most of the transmission occurring from June to November (Greenwood BM, 1987). Samples were collected in the dry (low transmission) season. The Gambian immune donors had no recent history of clinical malaria infection and were without parasites in their blood at the time of sampling. Non-immune (control) samples were obtained either from Danish donors (Jepsen S, and Axelsen NH; 1980) with no prior exposure to malaria or from Europeans (Bate et al., 1988) who had spent less than one month in the Gambia and who had been taking regular antimalarial chemoprophylaxis.

Lymphocyte proliferation assays were performed as described previously (Theander TG et al., 1986) (Riley, EM et al., 1988). Briefly, mononuclear cells (MNC) were separated by density centrifugation and stimulated with Purified Protein Derivative of tuberculin (PPD) (State Seruminstitute, Copenhagen, Denmark) (12 µg/ml) or with Phytohemagglutinin (PHA) (Difco Laboratories, Detroit, MI, USA) (10

μg/ml), affinity purified soluble antigens (37 μg/ml total protein), antigen 7 (75 μg/ml total protein) or control buffer (20 μl/well). Assays were performed in triplicate in roundbottomed microtiter plates and cultures were incubated for either 3 days (PHA) or 7 days (PPD and antigens) at 37°C in 5% CO_2. Proliferation was determined by ^{3}H-thymidine incorporation. Assays were performed in triplicate in roundbottomed microtiter plates and cultures were incubated for 7 days at 37°C in 5% CO_2. Proliferation was determined by ^{3}H-thymidine incorporation.

The results are expressed both as Δkcpm (kcpm of stimulated cells - kcpm of unstimulated cells) and as a stimulation index (kcpm of stimulated cells/kcpm of unstimulated cells). A kcpm > 1,0, and a stimulation index of > 2,5 was considered to be indicative of a positive response.

Of the 30 immune donors initially tested in this assay 19 were selected for testing with antigen 7 on the basis of a significant *in vitro* lymphoproliferative response to the affinity purified soluble antigens. 13 of these 19 immune donors responded to antigen 7.

Only two of the 28 control donors responded to the soluble antigen complex (Δcpm = 1869 and 1083, SI = 6,86 and 13,07) and only one control donor responded to the purified antigen 7 (Δcpm = 2509, SI = 5,43).

There was no significant difference between immune donors and controls in the response to PHA or to PPD (Wilcoxon ranksam Test, $P > 0.5$). Sera from some of the donors were tested by CIE for precipitating antibody to antigen 7. Of 11 malaria immune donors with a positive proliferative response to antigen 7, 5 had precipitating antibodies to this antigen. In addition, 2 of 4 malaria immune donors with no proliferative response to the antigen 7 also had precipitating antibodies against antigen 7.

EXAMPLE 6

Production of a protein part of antigen 7

Production of synthetic peptide immunogens

Synthetic peptides expressing parts of the antigen 7 polypeptide sequences may be produced. These peptides may be used for vaccination experiments with the aim of inducing protection against the antigen 7 mediated disease manifestations during a malaria infection. Furthermore, the synthetic peptides may be used as antigen for the detection of antibodies against antigen 7 (see Example 11).

The synthetic peptides may be produced by modifications of the solid phase peptide synthesis described by Merrifield (1963). The modifications employed should be adapted to the particular sequence which is to be synthetized. Briefly, in solid phase synthesis, the amino acid sequence is constructed by coupling an initial amino acid to a solid support, a resin, and then sequentially adding the other amino acids in the sequence by peptide bonding until the desired length has been obtained. As an example, coupling reactions may be performed using four equivalents of N-α-t-butyloxycarbonyl (Boc) amino acids with dicyclohexylcarbodiimide couplings. After synthesis the peptide is cleaved from the resin, the specific cleaving conditions depending on the sequence. As an example, the peptide may be cleaved with anhydrous hydrogen fluoride for 2 hours at 0°C and for 1 hour at -20°C using p-cresol as a scavenger.

The peptide may be purified under specific conditions depending on the sequence. The peptide could be purified by preparative high pressure liquid chromatography on a reversed phase column and freeze dried.

For use in vaccination and in ELISA, the peptide should be coupled to a carrier. For ELISA, a carrier should be chosen which possesses epitopes which under normal conditions are not recognized by human sera. Carriers for this purpose are human albumin, keyhole limpet cyanin or another suitable carrier. For vaccination purposes, the

carrier should possess strong T cell epitopes, most preferably the carrier should contain T cell epitopes expressed by the malaria parasite. Other possibilities include tetanus toxoid and purified protein derivative.

Coupling of the peptide to the carrier may be employed by several different procedures including bis-diazotized benzidine coupling, carboiimide coupling, glutaraldehyde coupling. As an example, 10 mg of carrier protein dissolved in phosphate-buffered saline (PBS), pH 7.5 may be preactivated by dropwise adding 50 µl of glutaraldehyde (0.06-1%) for 30 min on ice under constant stirring. Then 1 mg of peptide dissolved in PBS pH 7.5 may be added and coupling allowed to proceed overnight at 4°C. The mixture could then be dialyzed against PBS.

Peptides can also be polymerized into synthetic proteins by the addition of cysteines at the N and C terminals and glycine and proline as spacers. This polymer can be used without a carrier.

The immunization procedure with synthetic peptides may be carried out as described in Example 3, typically using 100 µg per injection for mice and 250 µg for rabbits. For humans immunization could be given into the triceps muscle on days 0, 20 and 45. Dosis could be 200 µg-2 mg and the synthetic peptide could be absorbed to $Al(OH)_3$, before inoculation.

EXAMPLE 7

Development of a vaccine for stimulation of immune reactivity against antigen 7

In accordance with the principles of the present invention, a vaccine against malaria could be based on substantially the entire isolated antigen 7 disclosed herein or on analogue thereof having favorable immune stimulating properties. Suitable analogues are epitopes of

antigen 7, i.e. B-cell or T-cell epitopes which could be identified as follows:

a) B-lymphocyte epitopes

To localize the B-lymphocyte epitopes of antigen 7, it is contemplated to follow five approaches.

1) to digest the native antigen with proteolytic enzymes.

2) to determine the amino acid sequence of antigen 7 and evaluate the antigenicity of the sequence of antigen 7 using suitable computer programmes such as, e.g. the Hopp and Woods analyses, the Lewitt analysis or the surfaceplot (Synthetic Peptides Incorporated).

3) to produce deletion mutants using the Erase-a-base system.

4) to produce synthetic peptides of predicted epitopes.

5) to produce antiidiotypic antibodies to antibodies reactive with antigen 7.

b) T-lymphocyte epitopes

To determine T-lymphocyte epitopes, the gene encoding the protein part of antigen 7 can be sequenced and analyzed with the AMPHI program (Margalit et al., 1987).

Among the epitopes predicted by this analysis, the ones which are the most preserved amongst geographically different isolates are the most interesting. These are determined as follows:

Oligonucleotides covering the nucleotide sequences encoding each of the predicted epitopes are hybridized to the DNA from several different isolates of *P.falciparum* and by the strength of the hybridization, the degree of conservation can be estimated.
The most conserved epitopes are produced as synthetic peptides and tested for T-lymphocyte proliferation properties and lymphokine secretion.

The thus characterized most efficient B- and T-cell epitopes are then combined, either on the nucleotide level to be produced in an organism or as amino acids in synthetic peptides. The combination of the

epitopes should be tested for the preservation of the properties of the separate epitopes, because the immune stimulating properties of the separate epitopes could be abolished by putting them close together due to interaction between the amino acids.

A possibility is to use tandemly connected units of combined epitopes. One way to achieve this is to connect subunits of epitopes by means of SS-bridges between peptides as described by (Patarroyo M.E. et al, 1988). Other ways of doing this is to couple the subunits at the nucleotide level and express a novel protein or peptide containing multiple repetitions of the epitopes.

The epitope(s) is/are formulated into a vaccine, e.g. as described in Example 3 above, and used for immunization in accordance with conventional methods, e.g. as described in Example 3 above.

A vaccine comprising B-cell epitopes as well as T-cells epitope would be advantageous as it would stimulate the β-lymphocytes as well as the T-lymphocytes thus at the same time giving rise to a protection against and/or prevention of clinical manifestations of a malaria infection as well as the infection itself.

EXAMPLE 8

Development of an anti-idiotypic antibody vaccine for stimulation of immune reactivity against antigen 7

Antigen 7 epitopes may be mimicked by idiotype structures on immunoglobulins. Therefore, immunoglobulins may be used instead of the original antigen in a vaccine for stimulating immune reactivity against antigen 7. This approach is especially advantageous if the critical epitopes on antigen 7 has a glycolipid nature. Idiotypes may also be recognized by T-lymphocytes, therefore an anti-idiotypic vaccine may stimulate both humoral and cell mediated immune responses.

Murine monoclonal antibodies directed against antigen 7 with the capability to neutralize pyrogenicity, TNF production, etc. of anti-

gen 7 are selected. Preferably, these monoclonal antibodies express idiotypes common to human antibodies reacting with antigen 7.

The variable domains of the selected monoclonal antibodies is used for immunization of BALB/c mice. Typically, 100 µg antibody (digested with papain) in 100 µl is mixed 1+1 with Freund's Complete Adjuvans and injected subcutaneously in mice three times with 2-week intervals. 100 µg antibody alone is injected intraperitoneally 3 days before the spleens are taken for hybridoma production. Hybridoma cell cultures are produced from immunized mice by standard procedures. Culture supernatants are tested for their capability to inhibit the binding of the monoclonal antibody used for immunization to antigen 7 (see Example 3) when tested in ELISA. Selected hybridoma cultures are used for the production of monoclonal antibodies.

Selected antiidiotypic antibodies are coupled to purified protein derivative (PPD), keyhole limpet hemocyanin (KLH) or another suitable carrier with glutaraldehyde. Preferably, the antibody is coupled to a carrier expressing T cell epitopes of the malaria parasite.

Mice are injected three times at 2-week intervals with 100 µg doses of the antibody-carrier conjugate suspended in Alumn or another suitable adjuvant. For humans, a dose of 1 mg is preferred. One week after the second and the third injection, the mice are bleeded and their sera are tested for antibody reactivity to antigen 7 by immunoblotting and CIE and thereafter for their blocking effect on the antigen 7 mediated stimulation of TNF production.

The selected anti-idiotypic antibodies is also used as antigen in an ELISA (see Example 11) for the detection of naturally antibodies reacting with the anti-idiotypic antibodies. Sera from individuals of different immune status should be tested.

EXAMPLE 9

Pyrogen test

Pyrogenicity of antigen 7 was measured in conventional rabbits (Ssc:CPH), weighing 2.4-3.4 kg, as prescribed in European Pharmacopoeia 3rd Edition, 1986. For each test, 1.5-3.0 ml of antigen 7 was diluted with a pyrogen-free 0.9% m/v solution of sodium chloride to make up 10 ml test solution. For each test, 3 rabbits were injected i.v., each with 3 ml solution. As described in European Pharmacopoeia, rectal temperatures were monitored at 30 min intervals from 90 min before injection and continued for 3 hours after injection of the test solution.

In one test, 1.5 ml of antigen 7 was incubated with 20 µl rabbit anti antigen 7 Ig before pyrogen testing.

It was shown that antigen 7 is pyrogenic in rabbits, giving a mean temperature rise of 1.3°C over a period of 180 min (Fig. 10). Incubation of the antigen with specific rabbit-derived antibody dimished the mean temperature elevation to 0.7°C.

EXAMPLE 10

Sero-epidemiology

Immune reactivity with sera

Malaria immune sera from Indonesia (51 of 53 tested), Liberia (50 of 66), the Gambia (7 of 8), Guinea Bissau (4 of 9), Sudan (14 of 46) and Mocambique (13 of 15) react with antigen 7 from the F32 isolate when tested by CIE.

Seroepidemiological studies of antibody reactivity to antigen 7

A study was conducted among malaria exposed children and adults in a rural area of the Gambia. The donors included a group of 136 Gambian children aged 3 to 8 years. The age group in which, in this particu-

lar community, protective immunity against the disease malaria seems to develop very rapidly.

In May 1988, children were recruited near the town of Farafenni on the northern bank of the Gambia river. Malaria transmission in this area is intensely seasonal with most transmission occurring from July to November.

A clinical examination, including palpation of liver and spleen was performed. A venous blood sample (5-10 mls) was collected. Thick and thin blood films were stained with giemsa and examined for malaria parasites. Haematocrit was measured and plasma reserved for serology.

At the beginning of the rainy season, all parasitaemic children received a curative dose of chloroquine plus Maloprim (pyrimethamine plus dapsone). All children were then visited once a week by a field worker. A health questionnaire was completed, and the child's axillary temperatures was measured. A finger prick blood sample was taken from all children with a temperature of 37.5°C or more. An episode of clinical malaria was recorded in children with a raised temperature and a positive blood film. These children were treated with chloroquine. All children were re-examined in November 1988. Any child with a positive blood film, or with acquired splenomegaly, in whom an episode of fever had not been recorded, was classified as asymtomatic infection (group 2/3). Acquired splenomegaly was defined as the presence of a palpable spleen in November, which had not been palpable in May, or an increase in spleen size of at least 2 cm during the period. Two other groups were defined, those without parasites and fever (group 0) and those with parasites and fever (group 1).

Blood samples were also collected from adults in November and a blood film was examined for the presence of malaria parasites.

Finally, blood samples were taken from children with an acute episode of malaria (about 4 days after the beginning of the attack) and 3 weeks after in the convalescent phase.

Fig. 12 shows the age related prevalence of precipitating antibodies to antigen 7. The prevalence increases until the age of 5, where it becomes constant with about 75% of the donors having strong reactivity to the antigen and 10% having a weak reactivity.

Fig. 13 shows the prevalence of antibodies to antigen 7 in the three different donor categories in the May and November sample. Group 0 without parasites lose their antibodies to antigen 7 during the rainy season and probably these children will become susceptible to the disease the next year (children in this area normally have one clinical attack of malaria each year). The two categories with parasites in their blood keep their antibodies and those children without fever seems to have their antibody response boosted during the wet season. This boosting was not recorded for any other soluble antigen in this donor group.

Finally, 26/37 children in the acute phase had antibodies to antigen 7 while 26/27 children had antibodies to antigen 7 in the convalescent phase. These results shows that a malaria attack boosted the response to antigen 7.

EXAMPLE 11

Use of native antigen 7 or an analogue thereof, e.g. a fusion protein, synthetic peptide or anti-idiotypic antibody, as the antigen in an ELISA aimed at the detection of antibodies directed against *Plasmodium falciparum*.

Antigen 7 or an analogue thereof from a solution containing 1 mg/ml in PBS, pH 7.4, can be used for the coating of an ELISA plate in amounts of 0,01 - 10 ug per well in a volume of 100 µl of a carbonate buffer, pH 9.6. The plates can then be left overnight at 4°C in a humid chamber. (Coating of the ELISA plates can alternatively be performed by incubation for 2 hours in the humid chamber at room temperature on an orbital shaker.) Washing is performed with a Technunc Immunowasher 12 using the washing buffer. Each well is flooded with buffer 4 times. After washing, the plate is emptied and 100 µl

of a conservation buffer applied to each well. The ELISA plate can now be stored for at least two months at 4°C. Serum from malaria infected and uninfected blood donors to be tested is diluted in a dilution buffer 1:200 and 100 µl is applied to each well. Each sample should be tested in two wells. The plate is then placed at room temperature on an orbital shaker for one hour. The washing will be performed as described above and all fluids in the ELISA plate removed. A porcine anti human IgG conjugated to horseradish peroxidase (Dakopatts P214 dilution 1:10.000) can then be applied to the well in a volume of 100 µl. (For detection of antibodies in species other than the human, the horseradish peroxidase conjugated antibody is directed against the Ig of the species in question.) The plate is incubated at room temperature for one hour on an orbital shaker, then washed and emptied as described above. 100 µl colouring buffer is applied to each well, and the plate is incubated for 1 min. The colouring buffer is removed from the wells, and 100 µl of colouring substrate is applied to each well followed by incubation for 20-30 min. The colouring process is stopped by applying 150 µl 1M sulfuric acid to each well. The optical density at 490 nanometers is measured on a Titertek ELISA reader.

EXAMPLE 12.

Antigen detecting ELISA using

a) monoclonal or monospecific antibodies

Mouse monoclonal antibodies against the antigen 7 or an analogue thereof (obtained by immunization with the antigen or analogue and subsequent production of a hybridoma) or rabbit monospecific antibodies against antigen 7 are purified using a protein A column according to standard procedures. The antibodies are diluted to approx. 1 ug/ml in carbonate buffer, pH 9.6. 100 µl is applied to each well and left in a humid chamber overnight at 4°C. Coating of the ELISA plate can alternatively be performed by incubation for 2 hours in the humid chamber at room temperature on an orbital shaker. The plate is then

washed as above. For later use, 100 µl of conservation buffer is applied to each well and the plate stored covered with alufoil at 4°C in a humid chamber.

Before use, the conservation buffer is removed from the wells. Antigen containg fluid is diluted 1:2 in dilution buffer and 100 µl supplied to each well. The plate is incubated for 1 hour at room temperature on an orbital shaker and washed as described above. A biotinylated rabbit polyclonal antibody against antigen 7 or an analogue thereof is applied to the well in a concentration of 1:100 (or another appropriate concentration) diluted in dilution buffer. The plate is left at room temperature for 1 hour on an orbital shaker and washed as described above. Streptavidin conjugated to peroxidase is applied to the well in a volume of 100 µl diluted 1:1000 and left on an orbital shaker for 1 hour at room temperature. Washing is performed as above. Colouring buffer is applied in a volume of 100 µl to each well, left for 1 min and removed. 100 µl of colouring substrate is applied to each well. After 20-30 min at room temperature, the colouring process is stopped by applying 150 µl 1M sulforic acid to each well. Optical density at 490 nanometer is measured on an ELISA reader.

b) Polyclonal antibodies

Rabbit polyclonal antibodies against antigen 7 or an analogue thereof (obtained by immunization with antigen 7 or an analogue therof) are purified from rabbit serum using an antigen 7 column, a column carrying an analogue of antigen 7 and/or a protein A column according to standard procedures. The antibodies are diluted to approx. 4 µg/ml in carbonate buffer, pH 9.6. 100 µl is applied to each well and left in a humid chamber overnight at 4°C. Coating of the ELISA plate can alternatively be performed by incubation for 2 hours in the humid chamber at room temperature on an orbital shaker. The plate is then washed as above. For later use, 100 µl of conservation buffer is applied to each well and the plate stored covered with tin foil at 4°C in a humid chamber.

Before use, the conservation buffer is removed from the wells. Antigen-containing fluid is diluted 1:2 in dilution buffer, and 100 µl supplied to each well. The plate is incubated for 1 hour at room temperature on an orbital shaker and washed as described above. A mouse monoclonal antibody against antigen 7 or an analogue thereof (obtained by immunization with antigen 7 or the analogue thereof and subsequent production of a hybridoma) is applied to the well in a concentration of 1:10 (or another appropriate concentration) diluted in dilution buffer. The plate is left at room temperature for 1 hour on an orbital shaker and washed as described above. Rabbit anti-mouse immunoglobulin conjugated to horseradish peroxidase (Dakopatts P260) is applied to the well in a volume of 100 µl diluted 1:1000 and left on an orbital shaker for 1 hour at room temperature. Washing is performed as above. Colouring buffer is applied in a volume of 100 µl to each well, left for 1 min and removed. 100 µl of colouring substrate is applied to each well. After 20-30 min at room temperature, the colouring process is stopped by applying 150 µl 1M sulfuric acid to each well. Optical density at 490 nanometer is measured on an ELISA reader.

EXAMPLE 13

Antigen measurement using competitive ELISA.

The wells of an ELISA plate n. 4-39454 from Technunc are coated with antigen 7 or an analogue therof in a dilution of 1-0,2 pg diluted with carbonate buffer, pH 9.6, to a total volume of 100 µl. The plate is left overnight at 4°C in a humid chamber. Coating of the ELISA plates can alternatively be performed by 2 hours of incubation in a humid chamber at room temperature on an orbital shaker. 50 µl of the material to be tested is mixed with an equal volume of dilution buffer containing the relevant antibody (obtained from any species or cell system by immunization with the antigen 7 or an analogue thereof) in dilutions of 1:200-1:6400, or another appropriate dilution. The mixture is put into the well and incubated for 1 hour at room temperature on an orbital shaker. Washing is performed as above. An

antibody against IgG of the species used in the first step, conjugated to horseradish peroxidase, is applied to each well in a concentration suitable for the demonstration of antibodies coupled to the coating antigen in the wells. The plate is left on an orbital shaker for 1 hour at room temperature and washed as described above. 100 µl of colouring buffer is applied to each well for 1 min, then removed, and 100 µl of colouring substrate is applied to the well. The colouring reaction is allowed to continue for 20-30 min, and then stopped with 150 µl, 1M sulforic acid. The optical density at 490 nanometers is measured on an ELISA reader.

An alternative design of the competitive ELISA is to coat the wells of the ELISA plate, as described above, with antibodies (obtained from any species or cell system by immunization with the native antigen 7 or an analogue thereof) affinity purified with antigen 7 or an analogue thereof as the ligand. A concentration of antibodies of 0.004 µg/ml to 400 µg/ml in carbonate buffer is used. 50 µl of the sample is mixed with 50 µl of a dilution of antigen 7 or an analogue in dilution buffer. A series of consecutive dilutions is used, the dilutions ranging from 1 mg/ml to 1 picog/ml in dilution buffer or another range of dilutions which is found appropriate considering the type of sample.

The visualization of antigen 7 or the analogue thereof bound to the antibodies in the well may be performed using antigen 7 or an analogue thereof labelled with a fluorescent molecule, an enzyme capable of hydrolyzing a substrate and thereby changing the absorbance of the substrate at a given wavelength, or any other principle. In case the analogue is a fusion protein, an antibody directed against the non-antigen 7 related part of the fusion protein may be used to detect the presence of the fusion protein. If the fusion protein for instance contains β-galactosidase or a part thereof, the fusion protein may be detected using an anti-beta-galactosidase antibody labelled itself or using another antibody conjugated to any labelling principle in the next layer.

EXAMPLE 14

Production of T-cell clones reactive with antigen 7

Peripheral blood mononuclear cells (PBM) may be prepared by Ficoll-Hypaque (Pharmacia, Uppsala Sweden) centrifugation from heparinized blood samples taken from clinically malaria immune individuals.

PBM is adjusted to a concentration of 2 x 10^6 cells/ml in RPMI-1640 medium supplemented with 2mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin and 10 % pooled AB serum and incubated with antigen 7 for 6 days at 37°C in 5% CO_2/air. The cells is washed twice and transferred to medium supplemented with 100 U/ml recombinant IL-2 (Roche, Nutley, NJ), and, after another 7 days, cloned by limiting dilution.

For cloning, T-cell blasts is seeded at 0.3 cell/well in Terasaki trays (Falcon Division, Becton Dickinson and co., Cockeysville, MD) in the presence of phytohemagglutinin (PHA-l) (Polysciences. Inc.. Warrington, PA) (2 µg/ml) and 10^4 allogeneic irradiated (2500 Rad) PBM and IL-2. After 1-2 weeks, it is examined microscopically for cell growth, and growing cells is expanded further in medium with PHA, allogeneic irradiated PBM and IL-2. Subcloning procedures is identical to that described above.

Epstein Barr Virus (EBV) transformed B-cells may be used as antigen presenting cells for the T-cell clones. PBM from the same donor used for making T-cell clones is transformed with EBV. 10^7 PBM is resuspended in 10 ml RPMI 1640 medium supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin and 10% fetal calf serum containing 30% supernatant of the EBV-producing marmoset cell line B95.8 and 600 ng/ml Cyclosporin A (Sandoz, Basle, Switzerland) and distributed in the wells of a flat-bottom 96 well microculture plate at 5 x 10^4/well. 1 x 10^4 irradiated (5000 Rad) EBV transformed B-cells is cultured with 2 x 10^4 cloned T-cells for test of lymphocyte proliferation performed as described in Example 5.

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CLAIMS

1. A substantially pure soluble antigen derived from the malaria parasite *Plasmodium falciparum* in its schizont stage capable of stimulating the production of cytokines, which antigen is a amphiphilic glycoprotein which in a crossed immunoelectrophoresis (CIE) has a position in the γ-globulin fraction and which has fractions of a molecular mass of 77, 61, and 59, kDa when tested by SDS-PAGE analysis under conditions substantially as described in Example 1, or an analogue thereof.

1.a. An antigen according to claim 1 which further comprises fractions of a molecular mass of 35, 33 and 16 kDa.

2. An antigen according to claim 1 or 1a, which has endotoxin-like properties.

3. An antigen according to claim 2, which stimulates the *in vitro* or *in vivo* production of monokines such as tumor necrosis factor (TNF) or interleukin-1.

4. An antigen according to claim 2, which stimulates the *in vitro* or *in vivo* production of lymphokines such as γ-interferon, interleukin-2, interleukin-4, and interleukin-6.

5. An antigen according to any of claims 1-4, which is pyrogenic.

6. A substantially pure soluble antigen derived from a malaria parasite capable of elicting antibodies which neutralize the production of cytokines *in vitro* or *in vivo*, or an analogue of said antigen.

7. An antigen according to claim 6, which is derived from a *Plasmodium* spp.

8. An antigen according to claim 7, which is derived from *Plasmodium falciparum*.

9. An antigen according to any of the preceding claims, which comprises a T-cell epitope.

10. An antigen according to claim 1, which is the 16 kDa fraction.

11. Substantially pure antigen 7 from *Plasmodium falciparum*.

12. A substantially pure antigen which is recognized by an antibody raised against or reactive with native antigen 7, or an analogue thereof.

13. An antigen as defined in any of the preceding claims or an analogue thereof for use as a profylactic or therapeutic agent.

14. The use of an antigen as defined in any of claims 1-13 or an analogue thereof for the manufacture of a medicament for the profylaxis or treatment of diseases caused by *Plasmodium* spp.

15. A substantially pure polypeptide derived from an antigen as defined in any of claims 1-13.

16. A recombinant polypeptide according to claim 15.

17. A synthetic polypeptide according to claim 15.

18. A polypeptide as defined in any of claims 15-17 for use as a profylactic or therapeutic agent.

19. The use of a polypeptide as defined in any of claims 15-17 for the manufacture of a medicament for the profylaxis or treatment of diseases caused by *Plasmodium* spp.

20. An anti-idiotypic antibody mimicking the structure of an antigen as defined in any of claims 1-13 or an analogue thereof and capable of stimulating T-cells and antibodies reactive with the antigen or analogue.

21. An anti-idiotypic antibody according to claim 20 for use as as a profylactic or therapeutic agent.

22. The use of an anti-idiotypic antibody as defined in claim 20 in the manufacture of a medicament for the profylaxis or treatment of diseases caused by *Plasmodium* spp.

23. A vaccine for immunizing an animal, including a human being, against diseases caused by a plasmodial parasite, the vaccine comprising an immunologically effective and physiologically acceptable amount of an antigen as defined in any of claims 1-13 or an analogue thereof together with a physiologically compatible carrier or vehicle.

24. A vaccine according to claim 23 for immunization against the clinical manifistations of a malaria infection.

25. A vaccine according to claim 23 or 24, in which the plasmodial parasite is *P. falciparum*.

26. A vaccine according to any of claims 23-25, which further comprises one or more antigens derived from *Plasmodium* spp. different from the antigen as defined in claims 1-13 or an analogue thereof.

27. A vaccine according to any of claims 23-26 which further comprises immunologically active molecules obtained from pathogenic organisms other than *Plasmodium* spp.

28. A vaccine according to any of claims 23-27, which further comprises an adjuvant.

29. A vaccine according to claim 28, wherein the adjuvant is selected from the group consisting of Freund's incomplete or complete adjuvant, aluminium hydroxide, a saponin, a muramyl dipeptide, a lipopolysaccharide, immunogenic parts of other microorganisms, a T-cell immunogen, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interferon-γ, an oil, such as a vegetable oil, e.g. peanut oil, or a mineral oil, e.g. silicone oil,

purified protein derivatives (PPD), and Bacille Calmet Guerin (B.C.G.).

30. A vaccine according to any of claims 23-29, wherein the antigen or analogue therof is incorporated into micelles.

31. A vaccine according to any of claims 23-30, wherein the antigen or analogue thereof is incorporated into iscoms.

32. A vaccine according to any of claims 23-31, wherein the carrier is a macromolecular carrier.

33. A vaccine according to claim 32, wherein the macromolecular carrier is a polysaccharide or a polypeptide.

34. A vaccine according to claim 31 or 32, wherein the antigen or analogue thereof is multivalently coupled to the macromolecular carrier.

35. A vaccine according to any of claims 23-34, wherein the antigen or analogue thereof is polymerized.

36. A vaccine according to any of claims 23-35 being in a form suitable for parenteral, e.g. subcutaneous, intracutaneous or intramuscular, administration.

37. A vaccine according to any of claims 23-36 being in a form suitable for oral, nasal or rectal administration.

38. A vaccine for immunizing an animal, including a human being, against diseases caused by a plasmodial parasite, the vaccine comprising an immunologically effective and physiologically acceptable amount of a polypeptide as defined in any of claims 15-17 or an antiideotypic antibody as defined in claim 20 or 21 together with a physiologically compatible carrier or vehicle.

39. A vaccine according to claim 38 for immunization against the clinical manifistations of a malaria infection.

40. A method of producing an antigen as defined in any of claims 1-13 or an analogue thereof comprising cultivating or breeding an organism under conditions leading to expression of said antigen and recovering the antigen from the organism.

41. A method according to claim 40, wherein the recovering of the antigen or analogue thereof involves chromatography, especially affinity chromatography employing an antibody raised against or being reactive with an antigen as defined in any of claims 1-13 or an analogue therof.

42. A method of producing an antigen as defined in any of claims 1-13 or an analogue thereof, the method comprising the following steps:

a) isolating a DNA fragment encoding an antigen as defined in any of claims 15-17,

b) inserting the DNA fragment obtained in a) in an expression vector,

c) transforming a suitable host cell with the vector produced in step b),

d) cultivating the cell produced in step c) under suitable conditions for expressing the antigen or analogue or a post-translationally modified antigen or analogue, and

e) recovering the antigen or analogue or the modified antigen or analogue from the culture.

43. A method according to claim 42, which further comprises subjecting the antigen or analogue or modified antigen or analogue to posttranslational modifications to alter the immunological or physiological properties or activities of the antigen or analogue or modified antigen or analogue.

44. A method according to claim 42, wherein the DNA fragment coding for the antigen or analogue is modified by substitution, addition, insertion, deletion, or rearrangement of one or more nucleotides in the fragment.

45. A method according to claim 40 or 42, wherein the organism is a mammalian cell line or a microorganism.

46. A method according to claim 40 or 42-45, wherein the organism is subjected to mutation.

47. A method according to any of claims 42-46, wherein the antigen or analogue is isolated from the culture by a method comprising one or more affinity chromatography and/or size chromatography steps, and optionally employing a step using an antibody reactive with the antigen or analogue.

48. A method of producing a polypeptide as defined in any of claims 15-17, the method comprising performing a liquid or solid phase peptide synthesis.

49. A microorganism according to claim 45, wherein the nucleotide sequence encoding an antigen as defined in any of claims 1-13 or an analogue therof is modified by substitution, addition, insertion, deletion or rearrangement of one or more nucleotides in the sequence.

50. A non-pathogenic microorganism which is suitable for use as a live vaccine for the immunization of an animal against diseases caused by plasmodial parasites, the microorganism carrying, and/or being capable of expressing, an inserted nucleotide sequence coding for an immunologically active part of the antigen as defined in any of claims 1-13.

51. The use of a non-pathogenic microorganism carrying, and being capable of expressing, an inserted nucleotide sequence coding for an immunologically active part of an antigen as defined in any of claims 1-13 for the preparation of a live vaccine for the immunization of an animal against diseases caused by plasmodial parasites.

52. A method of obtaining a protective immunity in an animal, including a human being, against diseases caused by plasmodial parasites, the method comprising administering to the animal an immunogenically effective amount of at least one antigen as defined in any of claims 1-13 or an analogue thereof.

53. A T-cell clone obtained from an animal, including a human being, which is reactive with an antigen as defined in any of claims 1-13 or an analogue thereof.

54. A polyclonal monospecific antibody wich is ractive with an antigen as described in any of the claims 1-13 or an analogue thereof.

55. A monoclonal antibody which is reactive with an antigen as defined in any of claims 1-13 or an analogue thereof.

56. A monoclonal antibody according to claim 55 which is produced by a hybridoma cell line, or by clones or subclones thereof or by cell carrying genetic information from the hybridoma cell line coding for said monoclonal antibody.

57. A method of producing a monoclonal antibody reactive with at least a part of an antigen as defined in any of claims 1-13 or an analogue thereof comprising

administering in an immunogenic form at least a part of an antigen as defined in any of claims 1-13 or an analogue thereof to a living organism or cells to obtain cells producing an antibody reactive with said antigen or part thereof,
and isolating the antibody containing material from the organism or the cells.

58. A method according to claim 57 which further comprises

fusing cells producing the monoclonal antibody with cells of a suitable cell line, and selecting and cloning the resulting hybridoma cells producing the monoclonal antibody, or

immortalizing an unfused cell line producing the monoclonal antibody, followed by growing the cells in a suitable medium to produce said antibody and harvesting the monoclonal antibody from the growth medium.

59. A method according to claim 57 or 58, wherein the immunized animal is selected from the group consisting of rabbit, monkey, sheep, goat, mouse, rat, pig, horse and guinea pig.

60. A method of producing anti-idiotypic antibody as defined in claim 20 or 21 comprising immunising an animal with an antibody as defined in claim 52, 54 or 55 and recovering the resulting anti-idiotypic antibody.

61. The use of an antibody as defined in any of claims 52 or 54-56 or a T-cell clone as defined in claim 53 in an assay for the identification and/or quantification of the antigen or analogue thereof present in a sample.

62. The use of an antigen as defined in any of claims 1-13 or an analogue thereof in an assay for the diagnosis of an infection with a *Plasmodium* species.

63. The use according to claim 62, wherein the sample is a specimen obtained from a living organism such as a human or an animal.

64. The use according to claim 63, wherein the specimen is blood, e.g. an erythrocyte enriched fraction, or a tissue sample e.g. comprising liver cells.

65. The use of an antigen as defined in any of claims 1-13 or an analogue thereof in an assay for the diagnosis of an infection in an organism with a *Plasmodium* species, the assay involving examination of an urine sample obtained from said organism.

66. The use according to any of claims 61-65 further comprising using

an antibody as defined in claim 52, 54 or 55 and/or an antibody produced according to the method of any of claims 57-59.

67. The use according to any of claims 61-66, wherein the antigen or an analogue thereof is provided with a detectable marker.

68. An antigen according to any of claims 1-13 or an analogue therof provided with a detectable label.

69. An antibody according to claim 53 or 54 and/or an antibody produced by the method according to any of claims 56-58 provided with a detectable label.

70. An antigen according to claim 68 or an antibody according to claim 69 wherein the label is selected from the group consisting of enzymes, fluorephores, radioactive isotopes and complexing agents such as biotin.

71. An antigen according to any of claims 1-13 or an analogue thereof or an antibody according to claim 52, 54 or 55 coupled to a solid support.

72. An antigen or an antibody according to claim 71 wherein the support is selected from the group consisting of plates, strips, beads, particles, film and paper.

73. An antigen or an antibody according to claim 72, wherein the solid support comprises a polymer, e.g. selected from the group consisting of plastics, e.g. latex, polystyrene, polyvinyl chloride, polyolefin, nylon or polyvinylidene difluoride, cellulose, silicone and silica.

74. A method of determining the presence of a *Plasmodium* species molecule in a sample, wherein the sample is incubated with a monoclonal or polyclonal antibody or a T-cell clone as defined in claim 53 coupled to a solid support and subsequently with an antigen as defined in any of claims 1-13 or an analogue thereof provided with a label, or wherein the sample is incubated with an antigen as defined

in any of claims 1-13 or an analogue thereof coupled to a solid support and subsequently with a monoclonal and/or polyclonal antibody provided with a label.

75. A composition for the passive immunization of an animal, including a human being, against diseases caused by plasmodial parasites, which comprises a monoclonal antibody according to claim 52, 54 or 55 or produced by the method of any of claims 57-59 or a T-cell clone as defined in claim 53 and a suitable carrier or vehicle.

76. A diagnostic agent for the detection of an antigen as defined in any of claims 1-13 or an analogue thereof, which comprises a labelled DNA sequence homologous with a DNA sequence coding for a polypeptide part of said antigen or analogue thereof.

77. A diagnostic agent according to claim 76, wherein the label is selected from radioactive isotopes, enzymes, chemical modifying agents such as sulphonyl-introducing compounds and complexing agents such as biotin.

78. A method of isolating an antigen as defined in any of claims 1-13 or an analogue thereof, the method comprising adsorbing a biological material containing said antigen or analogue thereof to a matrix comprising an immobilized monoclonal antibody according to claim 52, 54 or 55, eluting said antigen or analogue thereof from said matrix and recovering the antigen or the analogue thereof from the eluate.

79. A method of determining the presence in a sample of antibodies reactive with an antigen as defined in any of claims 1-13 or an analogue thereof, the method comprising contacting the sample with the antigen or the analogue thereof, and detecting the presence of bound antibody resulting from said contacting and correlating the result with a reference value.

80. A method for monitoring the amount of an antigen as defined in any of claims 1-13 or an analogue thereof in a vaccine, comprising contacting the vaccine with an antibody as defined in claim 52, 54 or

55 optionally provided with a label and detecting the amount bound antibody.

81. A method of treating the clinical manifestations of infection with a malaria parasite in a patient, comprising administering to the patient an immunologically effective amount of an antibody as defined in claim 52, 54 or 55 or a T-cell clone as defined in claim 53.

82. A method of vaccinating a human being against clinical malaria comprising adminstering an antigen as defined in any of claims 1-13 or an analogue therof to the patient.

Fig. 1

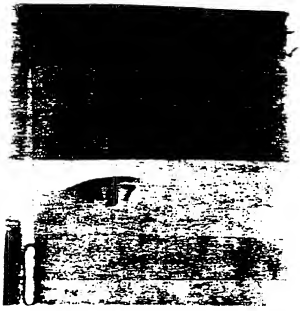
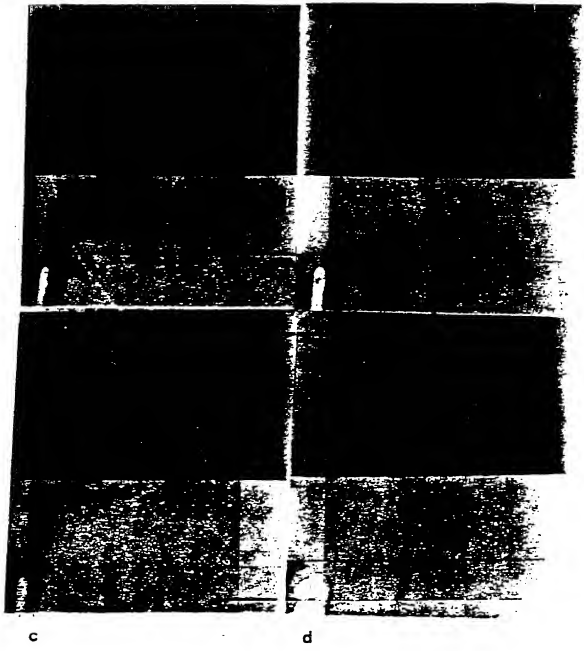


Fig. 2



ABSTRACT

The present invention relates to a substantially pure soluble antigen derived from the malaria parasite *Plasmodium falciparum* in its schizont stage capable of stimulating the production of cytokines, and analogues thereof. The antigen has endotoxin-like properties and stimulates the production of monokines and/or lymphokines, moreover, the antigen is capable of elicting antibodies which neutralize the production of cytokines. Furthermore, the present invention relates to antibodies which are reactive with the antigen or an analogue thereof and a method of determining the presence of a *Plasmodium* species molecule in a sample, the sample being incubated with e.g. an antibody which is reactive with the antigen or an analogue thereof, coupled to a solid support.

The present invention further relates to the use of said antigen or an analogue thereof for the manufacture of a medicament for the prophylaxis or treatment of diseases caused by *Plasmodium* species, and to a vaccine for immunizing an animal, including a human being, against diseases caused by a plasmodial parasite, the vaccine comprising an immunologically effective and physiologically acceptable amount of said antigen or an analogue thereof.

The antigen is an amphiphilic glycoprotein which in a crossed immunoelectrophoresis (CIE) has a position in the γ-globulin fraction and which has fractions of a molecular mass of 77, 61 and 59 kD when tested by SDS-PAGE analysis.

Fig. 3

a

b

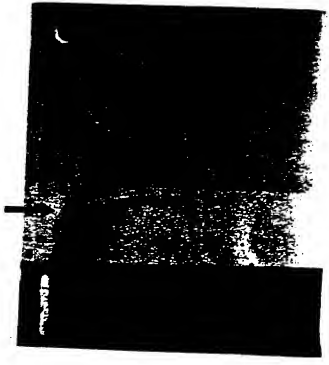


Fig. 4

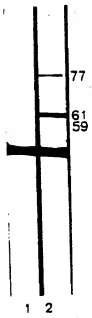


Fig. 5

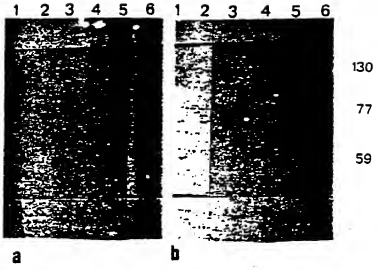


Fig. 6

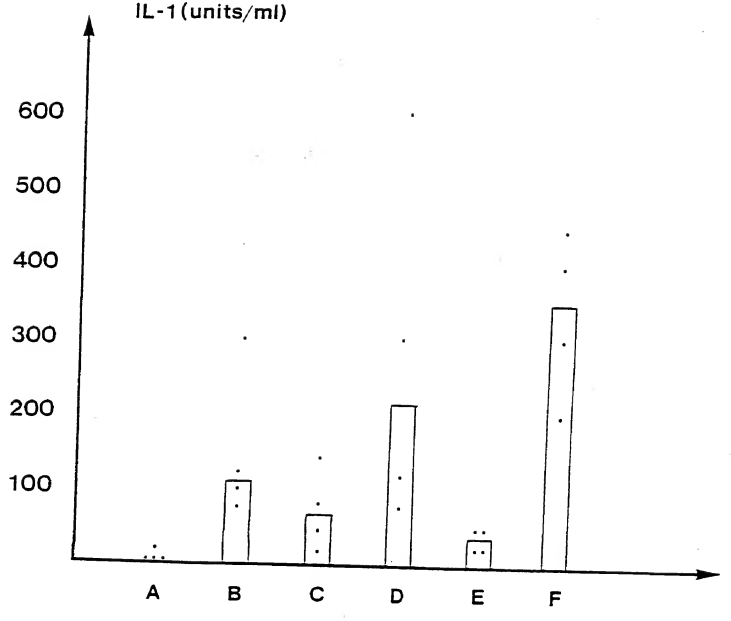


Fig. 7

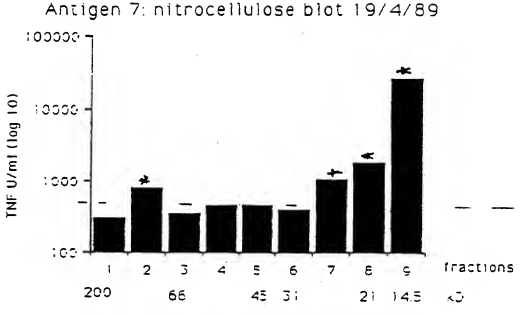

Antigen 7: nitrocellulose blot 19/4/89
TNF U/ml (log 10)
100000
10000
1000
100
1
2
3
4
5
6
7
8
9
fractions
200
66
45
31
21
14.5
<10

Fig. 8

Fig. 9

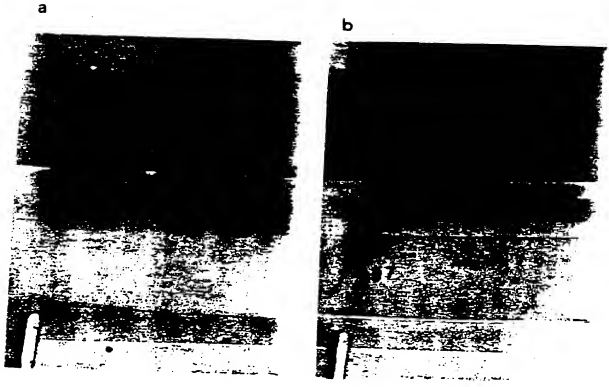
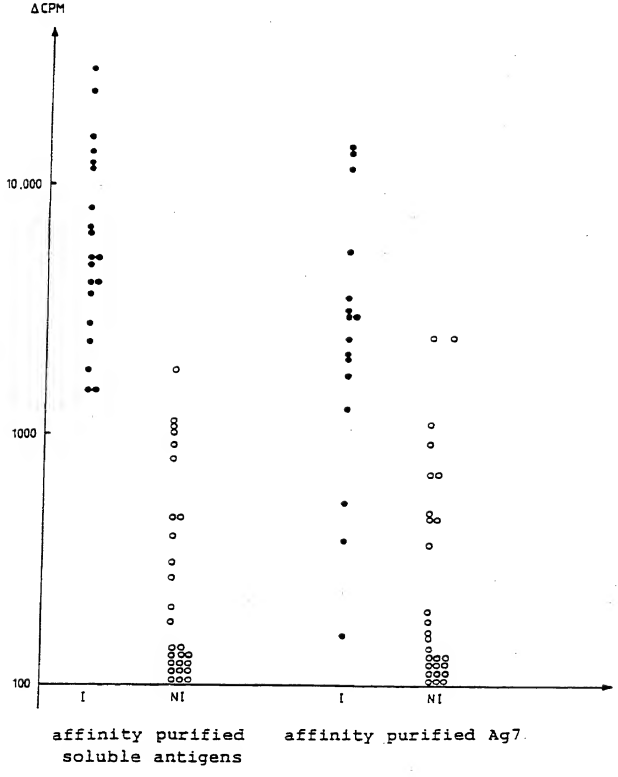


Fig. 10


ΔCPM
10.000
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NI
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NI
affinity purified soluble antigens
affinity purified Ag7

Fig. 11

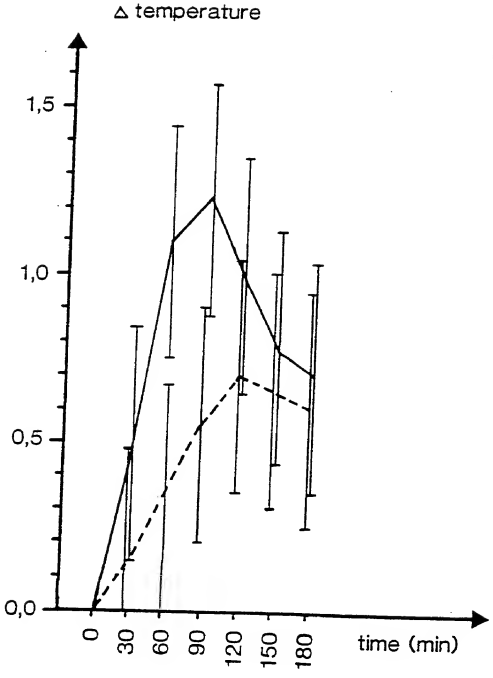


Fig. 12

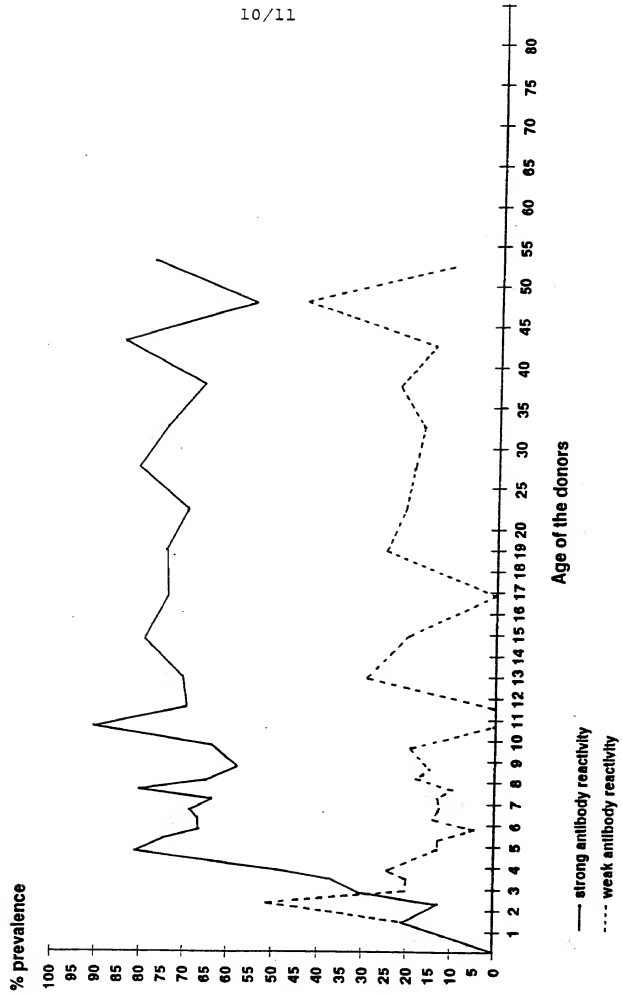
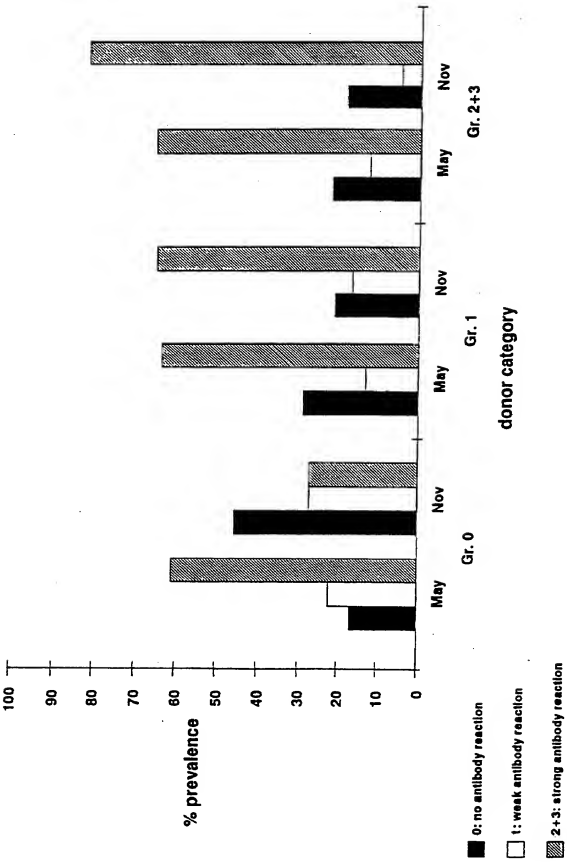

% prevalence
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 25 30 35 40 45 50 55 60 65 70 75 80
Age of the donors
strong antibody reactivity
weak antibody reactivity

Fig. 13


100
90
80
70
60
50
40
30
20
10
0
% prevalence
May
Nov
Gr. 0
May
Nov
Gr. 1
May
Nov
Gr. 2+3
donor category
0: no antibody reaction
1: weak antibody reaction
2+3: strong antibody reaction

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 90/00159

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: A 61 K 39/015, C 07 K 15/04, C 07 K 15/28, A 61 K 39/395, C 12 P 21/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System [8]	Classification Symbols
IPC[5]	A 61 K, C 07 K, C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [9]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Parasite Immunology, vol. 10, 1988, P.H. Jakobsen et al.: "Demonstration of soluble Plasmodium falciparum antigens reactive with Limulus a amoebocyte lysate and polymyxin B", pages 593-606, see the whole article, especially pages 594-596: "Materials and Methods"; pages 596-604: "Results" and Discussion"	1-19,23-51, 53-54,61-74, 76-77,79-80
Y		20-22,38, 55-60,75,78
	--	
Y	WO, A, 89/01785 (GEORGETOWN UNIVERSITY) 9 March 1989 see pages 12-29; pages 33-34, example I; page 50, line 15 - page 51, line 14; pages 71-74, claims	20-22,38, 55-60,75,78
	-- ./.	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17th October 1990	16. 11. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	[signature]

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	Parasitology Research, vol. 73, 1987, Springer-Verlag, P.H. Jakobsen et al.: "Inhibitory monoclonal antibodies to soluble Plasmodium falciparum antigens", pages 518-523, see front page, abstract; pages 518-520: "Materials and Methods"; pages 520-521: "Immunoblotting"; pages 521-523: "Discussion"	1-19,23-51, 53-59,61-80
A	EP, A, 0223665 (INSERM) 27 May 1987 see page 3, line 50 - page 4, line 2; pages 15-16, claims 1-14	1-19,23-51, 53-59,61-80
A	WO, A, 88/00597 (SARAMANE PTY, LTD) 28 January 1988 see the whole document	1.a.-19, 23-51,53-59, 61-80
A	WO, A, 89/05348 (SARAMANE PTY, LTD) 15 June 1989 see the whole document	1-19,23-51, 53-59,61-80
A	WO, A, 85/00977 (INSTITUT MERIEUX) 14 March 1985 see the whole document, especially page 6, lines 5-20	1,2-9,11-19, 23-51,53-54, 61-74,76-77, 79-80
A	Infection and Immunity, vol. 53, no. 1, July 1986, American Society for Microbiology, T.G. Theander et al.: "Proliferation induced by Plasmodium falciparum antigen and interleukin-2 production by lymphocytes isolated from malaria-immune individuals" pages 221-225, see the whole article	1-51,53-80

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers **, because they relate to subject matter not required to be searched by this Authority, namely:

** Claim numbers 52,81,82
See PCT Rule 39.1(iv):
Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [ ] Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers ....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. DK 9000159
SA 38153

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 08/11/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A- 8901785	09-03-89	AU-A-	2384688	31-03-89
EP-A- 0223665	27-05-87	FR-A,B	2589062	30-04-87
		JP-A-	62135499	18-06-87
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		EP-A-	0153939	11-09-85
		JP-T-	61500169	30-01-86